MOLECULAR CHARACTERIZATION AND DIFFERENTIAL EXPRESSION OF A INVERTASE GENE FAMILY IN MAIZE

By

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Abstract of Dissertation Presented to the Graduate School
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MOLECULAR CHARACTERIZATION AND DIFFERENTIAL EXPRESSION OF A INVERTASE GENE FAMILY IN MAIZE

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A family of soluble invertase genes in maize (*Zea mays* L.) were cloned and characterized to test several hypotheses regarding their potential significance in specific instances of developmental and/or environmental adjustment. The responses of two invertase gene subfamilies were examined at the level of both gene expression and overall enzyme activity.

Five maize cDNA clones (*Ivr1*, *Ivr2*, *Ivr2C-1*, *IvrC-2* and *IvrC-3*) and one genomic clone (*Ivr1G*) were isolated and found to encode probable isozymes of soluble invertase. The deduced amino acid sequences show significant identities, especially to previously characterized soluble acid invertases of higher plants, and are particularly strong in key regions conserved among these enzymes. One of the most strongly conserved regions among all invertase sequences (NDPNG) was found

to be carried on an unusually small 9 nucleotide exon identified in the maize genomic DNA.

Two subfamilies of maize soluble invertases (each cross-reactive with either *Ivr1* or *Ivr2* [*Ivr2C 1* + *Ivr2C-2*]) were differentially expressed in an array of tissues. A comparison between message and enzyme activity was consistent with both subgroups encoding soluble acid invertases. The spatial and temporal patterns of expression for the two invertase classes, as well as the contrast between them implicate their potential involvement in several stages of development. Data support the hypothesis that invertase could be especially important during stages requiring expansion of specific cells, such as during pollination and early kernel development.

Maize root tips were used to further test the extent to which expression of the two subfamilies for soluble invertase isozymes may have been regulated by sugar levels or specific developmental signals.

The mRNA levels from both subgroups were elevated in the presence of exogenous sugar supplies as long as these were readily metabolizable, however the extent of this response differed. The *Ivr2* group of genes showed a greater sensitivity to carbohydrate deprivation. The differential responsiveness of invertase gene subfamilies to carbohydrate availability provides a potential mechanism for different isozyme genes to predominate in various tissues developmental stages, and/or altered environmental conditions.

Data also indicated that specific developmental cues could affect expression of both invertase subgroups as well as soluble activity of acid enzymes. Cytokinin

signals (typically produced by dividing cell, endosperm, root tips and symbionts) could alone apparently replace and supersede the carbohydrate upregulation of invertase transcript levels by sugars. Both *Ivr1* and *Ivr2* type mRNA abundance was upregulated by exogenous ABA (elevated in developing seeds and in response to some stresses). However simultaneous presence of cytokinin appeared to be required before the ABA induced changes at the message-level could be transduced at the level of enzyme activity. The differential response of invertase isozyme genes to sugar levels and specific plant hormones suggests that integration of these types of signals may mediate developmental responses, symbiosis, and/or adaptation to stresses.

CHAPTER 1
INTRODUCTION

Sucrose is the most abundant long-distance transport carbohydrate in the plant kingdom. As such, it plays a central and vital role in plant growth and development. In vascular plants there are two known enzymatic reactions that can breakdown this sucrose. These are catalyzed by invertase and sucrose synthase.

Invertase is often considered an essential enzyme for carbohydrate metabolism and partitioning because of the nearly ubiquitous role of sucrose in photoassimilate translocation (Avigad, 1982; Hawker, 1985; Turgeon, 1989). This is supported by the observation that invertase deficient kernels of the *miniature 1* maize mutant develop abnormally in addition to their reduced size (Miller and Chourey, 1992). Further, primary root tips of another invertase deficient maize mutant, OH43, can not grow normally on sucrose agar (Robins, 1958; Duke et al. 1991).

Invertase activity is widely distributed within and among vascular plants. Several isoforms of invertase often can be present simultaneously in a given plant and/or organ. However, the roles of the individual isoforms are not well understood.

Early work with maize kernels (Shannon, 1972; Shannon and Dougherty, 1972; Doehlert and Felker, 1987; Doehlert et al. 1988) indicated that imported sucrose moved from phloem into the extracellular space where it was hydrolyzed by a cell-wall-bound, acid invertase. This was presumed to contribute to a sucrose

concentration gradient between the phloem and apoplast which facilitated translocation of sucrose into sugar-utilizing tissues. Although more recent studies have largely been consistent with this scenario they also indicate that the entire story may not be as simple (Shannon et al., 1993)

Rapidly expanding tissues require either invertase or sucrose synthase to convert sucrose to substrates necessary for respiratory and synthetic processes (Gauquinta, 1979, Avigad, 1982, Hawker 1985) Invertase can be especially important to cell expansion through generation of hexoses and their associated osmotic potential (Kaufman et al., 1973 Schmalstig and Cosgrove, 1988 1990)

Genes involved in metabolic pathways are often regulated by levels of metabolites (Carlson, 1987 Schuster, 1989 Maas et al., 1990 Koch et al 1992, Sadka et al 1994) In vascular plants, sugar responsive genes have been primarily characterized in storage organs (Rocha Sosa et al., 1989 Salanoubat and Belliard, 1989 Muller-Rober et al., 1990 DeWald et al., 1994 Sadka et al., 1994). However carbohydrate-induced changes in gene expression have also been identified for enzymes involved in photosynthesis (Sheen, 1990) and other metabolic pathways (Maas et al 1990 Koch et al., 1992, Graham et al., 1994) Data at the enzyme level have suggested that a similar sugar regulated gene expression may underlie responses of invertase to carbohydrate availability (Sacher et al., 1963, Glasziou et al 1966 Ricardo et al., 1972 Kaufman et al 1973) Together sugar-sensitivity of these sucrose-metabolizing genes may comprise a system for sensing and transducing signals of whole plant carbohydrate status

Plant growth regulators often have pleiotropic effects on plant growth and development. When an organism becomes more complex during its development, communication between its different part requires an appropriate signaling system In vascular plants, a complex hormonal system is largely responsible for signaling such a communication system (Lichtenga and Meantes, 1987) Invertase activity can be upregulated by abscisic acid, auxin, cytokinins and/or gibberellic acid in an array of vascular plants (Sacher et al , 1963, Glasziou et al 1966; Kaufman et al , 1973; Howard and Witham 1983, Ackerson, 1985 Weil and Rausch, 1990 Miyamoto et al , 1993 Wu et al., 1993).

The purpose of the present research was to test the hypothesis that regulation of two subfamilies of soluble invertase isozymes in maize could be consistent with a proposed role for these isozymes in specific aspects of cell expansion during development and/or environmental adjustment Specific objectives were as follows.

1 Clone and characterize maize invertase genes

2 Test the effects of altered carbohydrate availability on soluble invertase activity and abundance of mRNA from the two invertase subfamilies

3 Determine the extent to which changes in mRNA abundance and enzyme activity correspond to those proposed for given physiological roles of these isozymes in maize development.

4 Characterize the responsiveness of genes for invertase isozymes to developmental signals at both message and enzyme activity levels.

CHAPTER 2

LITERATURE REVIEW

Invertase and Its Functions

Invertase catalyzes one of the only two enzyme reactions known to breakdown sucrose into its constituent monosaccharides, glucose and fructose in vascular plants. Lack of this enzyme will result in abnormal growth and development of plants, which is evident in root tips of the Oh43 maize mutant (Robbins, 1958; Duke et al., 1991) and kernels of the *miniature-1* (Miller and Chourey 1992). Overexpression of invertase in leaves strongly influences growth and phenotype of transgenic plants (von Schaewen et al. 1990; Dickinson et al., 1991). Expression of an invertase gene also controls sugar composition in tomato fruit (Klann et al. 1993).

Invertase may be ubiquitous among multicellular plant species. Acid and neutral forms can be distinguished based on pH optima, and/or invertases can be classified as soluble vs cell-wall-bound forms (Avigad, 1982). An "alkaline" (neutral) invertase has recently been purified from sprouting soybean (Chen and Black, 1992). However, acidic invertases are generally more common and have been studied from an array of plants (Hauft and Jones, 1986a; Sturm and Chrispeels, 1990; Arai et al. 1991; Klann et al., 1992; Elliott et al. 1993; Ramloch-Lorenz et al., 1993; Schwebel-Dugue et al., 1994; Unger et al., 1994).

The physiological significance of invertase action has been debated over a considerable period of time. Work with sugarcane stems (Hawker and Hatch, 1965 Glasziou and Gayler, 1972) and corn kernels (Shannon, 1968 Shannon, 1972, Shannon and Dougherty, 1972) indicated that imported sucrose moved from phloem into the extracellular space where it was hydrolyzed by a cell-wall-bound, acid invertase This was presumed to contribute to a sucrose concentration gradient between the phloem and apoplast, enhancing the rate of sucrose transfer into sucrose utilizing tissues. More recent evidence is also consistent with the initial hypothesis (Lin et al , 1984 Doehlert, 1986 Doehlert and Felker 1987 Doehlert et al., 1988, Doehlert, 1990) Nonetheless, work done by other groups indicates that sucrose can be transported into *Zea mays* L. endosperm without invertase hydrolysis (Hitz et al., 1985 Cobb and Hannah, 1986, Schmalstig and Hitz, 1987, Cobb and Hannah, 1988) It is possible that sucrose can move into sucrose-utilizing tissues of maize kernels by both mechanisms

Soluble acid invertase activity is closely correlated with leaf expansion in bean (*Phaseolus vulgaris L.*) and *Citrus* whereas sucrose synthase activity is minimal and fairly constant (Morris and Arthur 1984 Schaffer et al , 1987) The authors suggest that soluble acid invertase activity is the primary enzyme responsible for sucrose catabolism in the expanding bean and citrus leaves Its activity is considered to be the primary determinant of sink potential in these systems

Invertase also predominates over sucrose synthase (which is barely detectable) in the earliest stages of development for maize kernels (Tsai et al. 1970

Chourey and Nelson, 1976 Chourey 1981) and snap bean pods (Sung et al., 1994) In addition, maize kernels induced to abort by high temperature have a much reduced activity of pedicel soluble invertase than do nonaborting kernels (Hanft and Jones, 1986a) The rapid expansion characteristic of this early development requires both osmotic constituents and substrates for respiratory and synthetic processes Soluble invertase can be especially important to cell enlargement through generation of hexoses and their associated osmotic potential (Kaufman 1973 Avigad, 1982, Schmalstig and Cosgrove 1988, 1990) Either invertase or sucrose synthase can provide an avenue for carbohydrate entry into respiratory and biosynthetic processes (Giaquinta, 1979 Avigad, 1982, Morris and Arthur, 1984b Hawker, 1985 Schaffer et al 1987)

Soluble invertase activity is also closely associated with other phases of reproductive development (Tsai et al., 1970, Jaynes and Nelson, 1971a, Shannon and Dougherty 1972 Singh and Knox 1984, Hubbard et al., 1989 Miroo and Schaffer 1991 Klann et al., 1992) Pryke and Berneir (1978) have found that increased content of sugar and activity of soluble acid invertase in the apices consistently appear to accompany the transition to flowering in *Sinapis alba* Invertase also appears to be involved in pollen function. The sucrose content from pollen grains of *Camellia japonica* decreases rapidly during growth of the tube Soluble invertase activity also increases during germination of cultured pollen and a high constant activity is found during the later stages of pollen tube growth (Nakamura et al 1980) Further, invertase is demonstrated to contribute to an *in vitro* chemotropism

of pearl millet pollen tubes toward stigmatic tissue through its production of glucose (Reger et al., 1992a, 1992b, 1993)

A number of different genes may be involved in these processes in vascular plants (Sturm and Chrispeels, 1990, Arai et al., 1992, Klann et al., 1992, Elliott et al., 1993, Ramaloch Lorenz et al., 1993, Schwebel-Dugue et al., 1994, Unger et al., 1994). In yeast, one gene encodes both cell-wall-bound and soluble invertases through differential splicing (Carlson and Botstein, 1982). However, in carrot at least seven different invertase genes have been distinguished (A. Sturm, personal communication)

Regulation of Invertase Expression

Invertase and Its Endogenous Inhibitors

Proteinaceous invertase inhibitors are found in an array of vascular plants (Pressey, 1966, 1967, 1968, Jaynes and Nelson, 1971b, Matsushita and Uritani, 1976, Bracho and Whitaker, 1990a, 1990b, Isla et al., 1992, Weil et al., 1994). In *Solanum tuberosum* they are located in the vacuole (Bracho and Whitaker, 1990a, 1990b, Isla et al., 1992) whereas in *Nicotiana tabacum* they are in the extracellular space (Weil et al., 1994). These inhibitors bind tightly and specifically to acid invertase and have molecular weights ranging from 17 to 23 KDa (Bracho and Whitaker, 1990b, Weil et al., 1994). However, it remains unclear by what

mechanism the endogenous inhibitors may regulate invertase activities either spatially or temporally

Plant Growth Regulators and Invertase

Invertase activity appears to be upregulated by abscisic acid, auxin, cytokinins and/or gibberellic acid depending on the system and tissues involved (Sacher et al., 1963, Glasziou et al 1966 Gayler and Glasziou, 1969; Kaufman et al , 1973, Howard and Witham, 1983 Morris and Arthur 1984 Ackerson, 1985 Schaffer et al 1987 Weil and Rausch, 1990 Miyamoto et al 1993, Wu et al 1993)

Both auxin and gibberellic acid stimulate cell enlargement, cell elongation and possibly phototropism and gravitropism (Davies, 1987 Kaufman and Song 1987 Kim et al , 1993 Wu et al 1993a, 1993b) The concentration of GA (gibberellic acid) which promotes growth closely parallels that which increases invertase activity in *Avena* stem segments (Kaufman et al 1973) The increased rate of hydrolysis of sucrose to hexose following the stimulation of acid invertase activity by GA is considered one means of generating an elevated level of osmotic constituents in the growing region of the stem (Morris and Arthur 1985) The stimulation of both invertase activity and stem growth by auxin is consistent with the finding that invertases are especially active in tissues undergoing rapid cell enlargement, such as regions near shoot and root apices (Avigad, 1982) The

mechanism by which IAA leads to an increase in acid invertase activity however remains obscure It is not yet clear whether the observed increase in activity is a cause or a consequence of auxin-induced growth (Morris and Arthur 1984a)

Both abscisic acid and cytokinins are reported to stimulate assimilate translocation from source to sink (Gerrath and Kender, 1982 Howard and Wilhite, 1983 Hein et al 1984, Schussler et al , 1984 Ackerson, 1985 Jones et al 1986 Brokovec and Prochazka, 1992, Jones et al , 1992) Such enhancement may involve the capacity of invertase to hydrolyze sucrose to hexoses and thus increase sink potential. This in turn could stimulate the translocation of sugar to seeds (Shannon, 1968, Shannon, 1972, Shannon and Dougherty 1972 Lin et al 1984, Doehlert, 1986 Doehlert and Felker 1987)

The mechanisms originally proposed to explain the effects of plant hormones on invertase have been questioned (Sacher et al , 1963 Glasziou et al 1966, Chrispeels and Varner 1967, Gayler and Glasziou 1969 Hagen et al , 1984) Gayler et al (1969) suggested that auxin and gibberellic acid may have aided stabilization of the mRNA for invertase They further suggested that the mechanism of abscisic acid (ABA) action in this instance involved processes subsequent to formation of invertase-mRNA and prior to invertase destruction In contrast, Chrispeels et al (1967) suggested that the gibberellic acid effect required synthesis of enzyme-specific RNA molecules They also proposed that abscisin exerted its action either by inhibiting the synthesis of these enzyme-specific RNA molecules or by preventing their incorporation into an active enzyme synthesizing unit

Most recent work at the molecular level, however, indicates that there are ABA-responsive elements and GA-responsive sequences located on promoter regions in a number of structural genes (Jacobsen and Beach, 1985; Zwar and Hooley, 1986; Libbenga and Mennes, 1987; Marcott et al., 1989; Mundy et al., 1990; Salmenkallio et al., 1990; Jacobsen and Close, 1991; Skriver et al., 1991; Lanahan et al., 1992). An auxin-responsive promoter appears to be differentially induced by auxin gradients during tropisms (Li et al., 1991). It is more likely that the effects of abscisic acid, auxin, cytokinin and/or gibberellic acid on invertase are mediated by their respective influence on transcription, but these may well occur by different mechanisms.

Wounding and Invertase

Wounding typically stimulates expression of invertase genes (Matsushita and Uritani, 1974; Sturm and Chrispeels, 1990). A general increase in the respiratory activity in response to wounding in various plant storage tissues is well documented (Matsushita and Uritani, 1974). In root tissue of sweet potato, respiratory activity doubles within 20 hours after wounding. The increased respiratory activity is paralleled by increases in RNA content and the *de novo* synthesis of enzymes (Shirras and Northcote, 1984). Invertase may well be one of these and would be advantageous in its enhancement of capacity to initiate sucrose breakdown.

Sugar Levels and Invertase

A number of recent reports demonstrate that various genes involved in metabolic pathways are either induced or repressed by sugars (Carlson, 1987 Schuster 1989 Maas et al., 1990 Sheen, 1990 Koch et al , 1992 Sadka et al., 1994) Studies of carbohydrate assimilation in potato tubers have revealed that genes encoding patatin (Rocha-Sosa et al., 1989) sucrose synthase (Salanoubat and Belliard, 1989) and ADP-glucose pyrophosphorylase (Muller-Rober et al., 1990) can all be induced by elevated levels of sucrose Similarly, the tuberous root storage protein genes of sweet potato (Hattori et al., 1990) and the vegetative storage protein genes (vegetative storage proteins, Sadka et al., 1994) are upregulated by sugars In *Arabidopsis*, sucrose mimics the light induction of nitrate reductase gene transcription (Cheng et al. 1992) In maize, elevated carbohydrate levels regulate the sucrose synthase genes differentially such that *Sus1* is stimulated whereas *Sh1* is repressed (Maas et al. 1990 Koch et al 1992)

Repression of gene expression by sugars has also been shown for other plant genes In maize mesophyll protoplasts seven photosynthetic genes are downregulated by photosynthetic end products sucrose and glucose and by the exogenous carbon source acetate (Sheen, 1990) In tobacco the glutamate dehydrogenase gene is suppressed by feeding glucose (Maestro et al., 1992), and in cucumber genes encoding enzymes of the glyoxylate cycle (malate synthase and isocitrate lyase) are repressed by carbon catabolites (Graham et al , 1994) Together,

these mechanisms may comprise a means for sensing and transducing signals of whole plant carbohydrate status, and subsequently altering plant metabolism and/or development.

Particularly important in such a possibility are the genes encoding those enzymes which can break down sucrose, sucrose synthase and invertase. Sugar responsiveness of the former has been characterized (Salanoubat and Belliard, 1989; Maas et al. 1990; Koch et al., 1992). Although invertase clones have not been previously characterized, levels of these enzymes appear to show a long term, carbohydrate responsiveness (Sacher et al., 1963; Glasziou et al., 1966; Ricardo et al. 1972; Kaufman et al., 1973; Sarokin and Carlson, 1984).

Kaufman et al. (1973) demonstrated that increased levels of invertase were correlated with the sustained growth of *Avena* stem segments in the presence of sucrose. Their data further indicated that the presence of sucrose greatly enhanced the GA effect on elevation of invertase activity. They suggested that substrate may stabilize the enzyme and/or aid its induction. Their studies also support the view that gibberellic acid, as well as substrate (sucrose) and end products (glucose and fructose), play a significant role in regulating invertase levels in *Avena* stem tissue. Moreover, such regulation could provide a mechanism for increasing the level of soluble saccharides needed for gibberellic acid-promoted growth.

However, Morris and Arthur (1984a) documented a drop in concentration of hexose sugars in internodal segments of *Phaseolus vulgaris* during incubation in the presence of auxin. The greatest decline in hexose concentrations occurred when

segments were treated with compounds which stimulated the most growth. They also suggested that by reducing sucrose concentrations in the apoplast and/or symplast of sink tissues, the acid invertases located in these respective compartments may contribute significantly to maintenance of source-to-sink gradients in sucrose concentration and hydrostatic pressure which drives phloem transport.

Fungi, Bacteria, and Invertase

Increased invertase activities have been reported in tissues of several plants infected by biotrophic fungi and/or bacteria (Callow and Lang, 1973, Laog et al 1975 Billett et al 1977 Billett and Burnett, 1978, Callow et al 1980 Krishnan and Pueppke, 1988 Sturm and Chrispeels, 1990,) In addition, a common feature of biotrophic fungal infections of vascular plants is an increased translocation of photosynthetic assimilates into infected plant parts, which is typically accompanied by accumulation of one or more host carbohydrates (Callow and Lang 1973 Laog et al , 1975 Billett et al 1977 Billett and Burnett, 1978 Callow et al 1980)

Billett et al (1977) have shown that infection of maize by the corn smut, *Ustilago maydis* stimulates assimilate movement into and accumulation of soluble sugars, and starch, in tissues. Smut in maize kernels results in rapid growth, cell division, and elevated rates of respiration Enhancement of maize invertase activity in these regions could facilitate competition with other sinks for the sugars needed to support these processes. Sucrose import and unloading from phloem could be

accelerated by a greater capacity for invertase to remove this sugar from the terminal end of the transport path

Little research, however has been directed toward understanding the mechanisms by which invertase activity is elevated in response to pathogens (Callow and Ling, 1973, Long et al., 1975, Billett et al., 1977, Billett and Burnett, 1978 Callow et al., 1980 Heidecker and Messing 1986 Collinge and Slusarenko 1987, Sheridan, 1988 Sturm and Chrispeels, 1990) The origin of the induced invertase protein (fungal vs host) has remained controversial (Billett et al., 1977, Callow et al 1980)

Agrobacterium tumefaciens and *Pseudomonas syringae* pv *Savastanoi* however, contain genes that specify the biosynthesis of cytokinin and indoleacetic acid (Morris, 1986 Morris, 1987 Ishikawa et al 1988, Weil and Rausch, 1990) Cytokinins and/or cytokinin-like substances are also reported to be synthesized in mycorrhizal fungi (Miller, 1967 Crafts and Miller, 1974, Ng et al 1982) and *Bradyrhizobium japonicum* (Sturtevant and Taller 1989) Allen et al (1980) found that cytokinin levels increase in the host plant following infection by vesicular-arbuscular mycorrhizae. Elevated levels of IAA and/or cytokinin have also been implicated in maize tissues infected by *Ustilago maydis* (Turian and Hamilton, 1960 Billett et al., 1977, Billett and Burnett, 1978).

Upregulation of invertase expression by fungal infection could facilitate enhancement and/or establishment of a symbiosis by providing hexoses for those fungi unable to metabolize sucrose. A resulting question is whether or not plant

hormones, such as IAA and/or GA, act as signals to target the elevation of invertase in plant parts infected by certain biotrophic fungi and/or bacteria (Heidecker and Messing, 1986; Morris, 1986; Collings and Slusarenko, 1987; Davies, 1987; Lubberga and Mennes, 1987; Morris, 1987; Ishikawa et al., 1988; Sheriden, 1988; Weil and Rausch, 1990)?

Sturm and Chrispeels (1990) imply that the homology between extracellular carrot β-fructosidase and the levan hydrolyzing enzyme, levanase, may allow carrot β-fructosidase (invertase) to hydrolyze the bacterial slime coat. In this way, invertase action could inhibit bacterial growth directly or make the pathogen susceptible to further defense reactions. In this scenario, invertase would function in a positive, protective role as a new and unrecognized pathogenesis-related protein.

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF MAIZE INVERTASE GENES

Introduction

Only two avenues known for enzymatic breakdown of sucrose exist in vascular plants. One is catalyzed by sucrose synthase the other by invertase. Two distinct types of invertase activities are found in plants (Avigad, 1982). One class has an optimum pH of 4.5 to 5.0 and includes acid invertases. The second class hydrolyzes sucrose at a maximal rate at pH 7.5 to 8.0 and is designed as the alkaline invertases. The existence of these two types of β-fructosidase is evident in many plants and/or organs (Avigad 1982). Acid invertases are located either inside the vacuole (soluble form) or in the extracellular space (varying degrees of soluble and cell-wall bound forms). In contrast, alkaline invertases are compartmentalized in cytoplasm (Hawker 1985).

Invertase genes encoding cell-wall and vacuolar (soluble) acid invertases have been characterized from carrot (Sturm and Chrispeels, 1990, Ramloch-Lorenz et al., 1993, Unger et al. 1994) tomato (Klann et al., 1992 Elliott et al., 1993), mung bean (Arai et al., 1992) and *Arabidopsis* (Schwebel Dugue et al. 1994).

In the present study, a tomato invertase clone (Klann et al 1992) was used to isolate an invertase cDNA from maize and this, in turn, was used to obtain additional maize clones. These findings provide the tools for further investigation along two lines. The first of these will be aimed at combining an analysis of sugar-responsiveness of these genes with that of the sucrose synthases, to define carbohydrate regulation of two different avenues for sucrose breakdown. The second will be to further clarify the potential functional significance for soluble invertase isozymes in development and/or environmental adjustment in maize.

Materials and Methods

Probe for cDNA Library Screening

A 0.45 Kb fragment from the 5'-end of a cDNA encoding a soluble acid invertase in tomato (Klann et al. 1992) was isolated from tomato clone and subcloned into a pUC19 vector. The recombinant plasmid was amplified in *E. coli* cells, purified, and used to screen a maize cDNA library (Sambrook et al., 1989).

cDNA Library Screening

A maize root tip cDNA library (λgt 10 Clontech, Palo Alto CA) was screened with the 0.45 kb tomato invertase cDNA fragment. One positive clone

containing a 1 2 kb cDNA was obtained. This maize fragment was subcloned and used to probe for further cDNAs from the same library. Twelve positive clones ranging from 0 5 to 2.2 kb were identified

Genomic Library Screening

A maize genomic fragment containing 8 kb DNA was identified by screening a genomic library (EMBL 3, Clontech, Palo Alto CA) with the 1 2 kb maize invertase cDNA clone

Hybridization with DNA probe

Procedures for library plating and production of filter replicas were conducted as recommended by Clontech (Palo Alto CA). Plaques or colonies were blotted to nylon membranes, and DNA was denatured in situ with NaOH (0 5 M) neutralized with Tris buffer (1 0 M, pH 7 5) and fixed by baking (80 °C 0 5-2 hr) (Sambrook et al., 1989). Filters were hybridized at either 50 °C (low stringency) or at 65 °C (high stringency) in a solution with the selected cDNA, 7% SDS, 250 mM Na_2HPO_4 (pH 7 2) and 1% BSA (Church and Gilbert, 1984). Tomato and/or maize invertase cDNA fragments were radiolabeled by random primer (BRL, Gaithersberg, MD). Blots were washed as described by Church and Gilbert (1984) and exposed to X-ray film with intensifying screens at -80 °C

DNA Sequencing

Selected cDNA and genomic DNA fragments were subcloned into a pUC 19 vector. The recombinant plasmids were amplified in *E. coli* cells and purified through $CsCl_2$ ultracentrifugation and/or with the use of QIAGEN-tip (QIAGEN Inc., Chatsworth, CA). Both strands of each cDNA and genomic DNA were sequenced by the Sequence Core Lab of ICBR (Interdisciplinary Center for Biotechnology Research) located at the University of Florida.

Analysis of DNA and Protein Sequences

Computer-assisted analyses of DNA and protein sequences were carried out with Geneworks (Release 2.2 IntelliGenetics, Inc., Mountain View, CA)

Results

One positive clone containing a 1.2 kb maize cDNA (*Ivr1*) was obtained (Figure 3-1) when a cDNA fragment encoding a soluble acid invertase in tomato (Klann et al., 1992) was used to screen a maize root tip cDNA library (λgt 10, Clontech, Palo Alto, CA).

This 1.2 kb maize fragment was used to rescreen the same library. Twelve positive clones ranging from 0.5 to 2.2 kb were identified. Sequences obtained from the longest of these indicated that none of them included a full-length cDNA clone. For this reason, a *HindIII-EcoRI* fragment from the longest clone (2.2 kb; Figure 3-1) was used to rescreen the library a second time. Seven positive clones were identified.

From the total of twenty clones, five were selected for full length sequencing based on their sizes, hybridization characteristics, and location of restriction sites (Figure 3-1). Sequence was provided by the Sequence Core Lab of the ICBR at the University of Florida. They were designated *Ivr1* through *Ivr2C-3* (Figure 3-1). *Ivr2C-2* was identical to *Ivr2*, and *Ivr2C-3* contained the same but shorter sequence as *Ivr2C-1*.

Further information was sought in the corresponding genomic sequence. A maize seedling genomic DNA library (EMBL 3, Clontech, Palo Alto, CA) was screened with a 1 kb *KpnI-EcoRI* fragment from the *Ivr1* cDNA (Figure 3-1, 3-7). One positive genomic clone was isolated and characterized. This clone consisted of

ca 8 kb DNA. Digestion with BamHI and KpnI generated three fragments, each of which was subcloned and sequenced.

The invertase coding region was deduced according to the information from cDNAs, recognition sites for intron splicing (Goodall and Filipowicz, 1989, 1991) and/or comparision with other invertases from vascular plants (Figure 3-5, 3-6). The gene for maize invertase 1 (*Ivr1G*) was organized into seven exons and six introns, as diagrammed in Figure 3-2A. The second exon was only 9 nucleotides long (Figure 3-4B), and has also been reported in tomato fruit vacuolar invertase (Elliott et al., 1993). The amino acids encoded by this 9 bp exon are located in a highly conserved domain found in all invertases cloned thus far (NDPNG, the β-fructosidase motif; Sturm and Chrispeels, 1990; Figure 3-2B).

The genomic DNA (*Ivr1G*) is almost identical to the *Ivr1* cDNA clone at the level of amino acid sequence, except for a few amino acid replacements. Genomic and cDNA clones are from different maize lines, *Ivr1G* being isolated from a B73 genomic library, and the *Ivr1* cDNA from a 'Merit' root cDNA library.

The deduced amino acid sequence from *Ivr1G* consisted of 670 residues (Figure 3-3) which predicted a molecular weight of 71,942 and an isoelectric point of 7.5. This protein also included five potential glycosylation sites (N-X-S/T): N^{65}, N^{275}, N^{518}, N^{595} and N^{639} (Figure 3-3). The amino-terminal sequence of the *Ivr1G* protein indicated a hydrophobic region between basic N and polar C terminals (Figure 3-3, 3-4) and other characteristics typical of a signal peptide ([-3, -1] rule von Heijne, 1986; K. Cline, personal communication). The predicted excision site for

the signal sequence according to the (-3, -1) method of von Heijne (1986) was between A^{73} and G^{74}

A comparison between the invertase genes isolated from maize in the present work and other invertases from vascular plants (Table 3-1, Figure 3-6), *Ivr1G* shared an approximate 60% amino acid identity with soluble invertases (Arai et al., 1992, Klann et al., 1992, Elliott et al. 1993, Unger et al., 1994) and 40% with insoluble forms (Sturm and Chrispeels, 1990, Ramloch-Lorenz et al., 1993), moreover the conserved key domains (NDPND [β-fructosidase motif, Sturm and Chrispeels, 1990], as well as FRDPTTA, TGMWEC and YASKTF) (Figure 3-5). In addition, the maize invertase gene has a significantly greater amino acid identity to the soluble isoforms of invertase than to the cell-wall-bound ones found in other vascular plants, especially at the C-terminus of this protein (Figure 3-6).

Restriction maps of invertase cDNA and genomic clones from maize are shown in Figure 3-1. *Ivr2* was found to share a 53% sequence similarity at the amino acid level to *Ivr1G* (Table 3-1), especially have extensive sequence similarity located at conserved domains (data not shown). The *Ivr1* probe (1 kb KpnI EcoRI fragment) did not cross-hybridize with the *Ivr2* or *Ivr2C 1* cDNAs at high stringency (Figure 3-7B). The *Ivr2* probe (200 bp PstI PstI fragment) cross reacted with the *Ivr2C 1* cDNA but not that of *Ivr1* (Figure 3-7C).

Ivr1 was missing its 3' end and contained one 5' unspliced (putative) intron (according to the sizes of 5' RACE products, E. Bihn unpublished data). *Ivr2*

contained an unusual 5 end, missing the NDPNG (sequence indicates possible incomplete intron splicing) *Ivr2C-1* was lacking the 3 end of its coding sequence

Table 3-1 Percentage comparative sequence similarity shared at the amino acid level between genomic and cDNA clones for maize soluble invertases (*Ivr1G* and *Ivr2*) and those of other invertases from vascular plants

Amino Acid Identity (%)

	Ivr1G	*Ivr2*
Tomato[a] (S)[b]	61	48
Mung Bean[a] (S)	59	56
Carrot[d] (SI)	59	49
Carrot[d] (SII)	59	49
Carrot[e] (CWI)[f]	42	32
Carrot[e](CWII)[f]	45	32
Ivr1G	100	53
Ivr2	53	100

[a]Tomato soluble invertase (Klann et al., 1992)
[b]S represents soluble isoform for invertase
[c]Mung bean soluble invertase (Aru et al., 1992).
[d]Carrot soluble invertases (Unger et al 1994)
[e]Carrot insoluble invertases (Sturm and Chrispeels, 1990, Sturm, unpublished data)
[f]CW represents cell wall-bound (insoluble) invertase.

Figure 3-1 Restriction maps of *Ivr* clones for maize soluble acid invertases
Restriction maps of maize soluble invertases (*Ivr1C*, *Ivr1* *Ivr2* and *Ivr2C 1*) Sites on the restriction maps are as follows. B BamH I, H Hind III, K, Kpn I P Pst I S Sma I

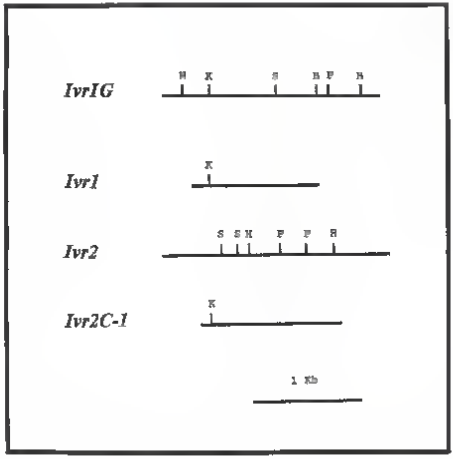

IvrIG
H K S B F N
Ivr1
K
Ivr2
S S K P P H
Ivr2C-1
K
1 Kb

Figure 3-2 Schematic diagram of the genomic organization of the *Ivr1G* gene for soluble invertase from *Zea mays* L. A. The entire *Ivr1G* gene for soluble invertase and bordering regions is depicted with exons as solid boxes. The locations of a putative CAAT box, TATA box, translation start (ATG), translation stop (TGA) are designated with arrows. B. Enlarged area from A, which encodes the most strongly conserved region among all invertase sequences (NDPNG, the β-fructosidase motif) (Sturm and Chrispeels, 1990)

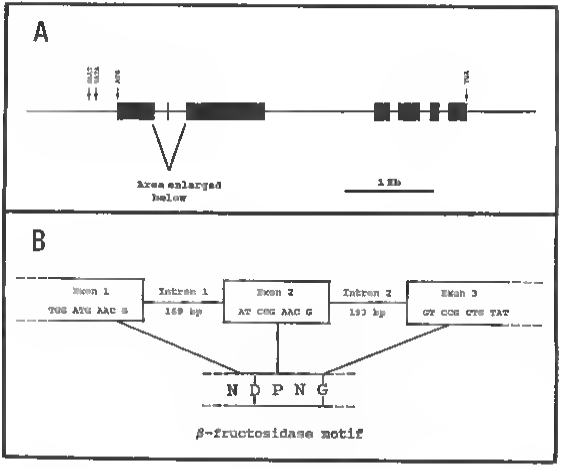
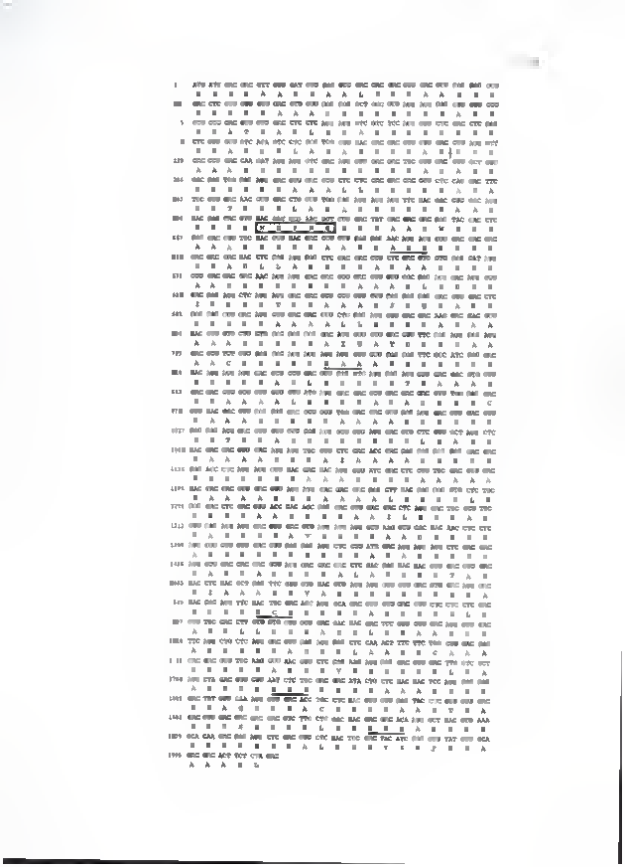

A
Area enlarged
below
1 Kb
B
Exon 1
TGG ATG AAC G
Intron 1
169 bp
Exon 2
AT CGG AAC G
Intron 2
193 bp
Exon 3
GT CCG CTG TAT
N D P N G
β-fructosidase motif

Figure 3-3 The deduced amino acid sequence for maize invertase 1 gene. The arrow indicates the cleavage site for potential signal peptide The box represents β fructosidase motif (NDPNG Sturm and Chrispeels, 1990) Underlined sequences are for putative glycosylation sites (N X S/T)



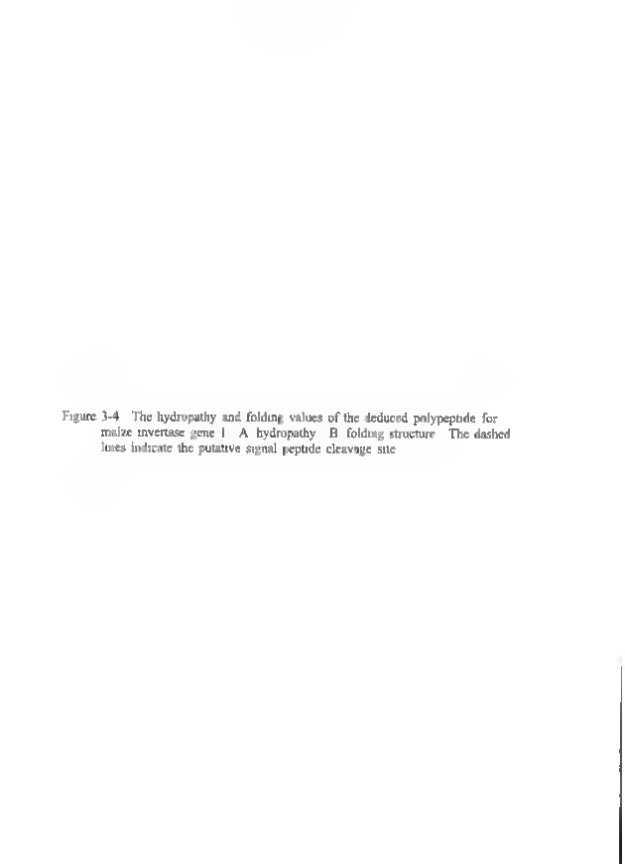


Figure 3-4 The hydropathy and folding values of the deduced polypeptide for maize invertase gene I A hydropathy B folding structure The dashed lines indicate the putative signal peptide cleavage site

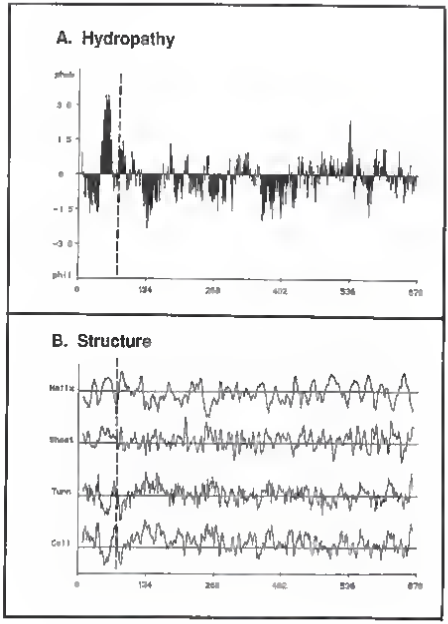

A. Hydropathy
phob
3.0
1.5
0
-1.5
-3.0
phil
0
134
268
402
536
670
B. Structure
Helix
Sheet
Turn
Coil
0
134
268
402
536
670

Figure 3-5 Conserved regions within derived amino acid sequences of higher plant invertases, shown here for the *Ivr1G* for maize soluble acid invertase gene 1, a mung bean soluble invertase (Arai et al., 1992) a tomato soluble invertase (Klann et al., 1992) two carrot soluble invertases (Unger et al., 1994), and two carrot insoluble invertases (Sturm and Chrispeels, 1990 Sturm, unpublished data) Boxes represent highly conserved regions

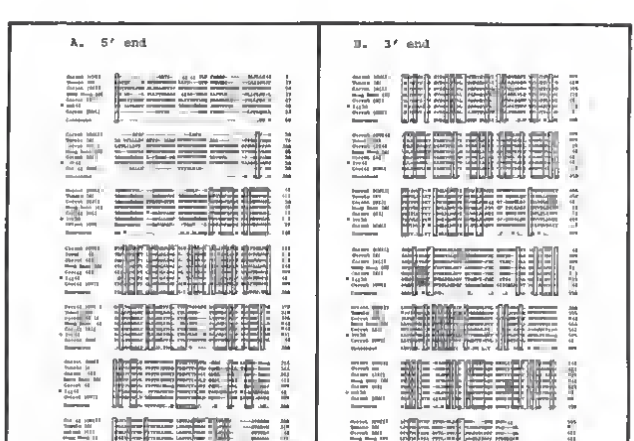
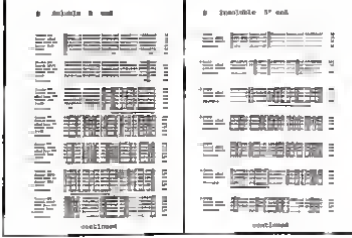

A. 5′ end
B. 3′ end

Figure 3 6 Conserved regions within derived amino acid sequences of the *Ivr1G* for maize soluble acid invertase gene 1 and either A other soluble invertases or B, insoluble invertases from other vascular plants. A Derived amino acid sequence similarities shared between the *Ivr1G* cDNA clone for maize soluble invertase, mung bean soluble invertase (Ara et al., 1992) tomato soluble invertase (Klann et al 1992) and carrot soluble invertases (Unger et al., 1994) B Derived amino acid sequence similarities shared between the *Ivr1G* and the insoluble invertases of carrot (Sturm and Chrispeels 1990 Sturm unpublished data) Boxes in A and B represent the most highly conserved regions and underlined sequences are those shared among soluble but not insoluble invertases.


B Soluble B end
B Insoluble B end
continued
continued

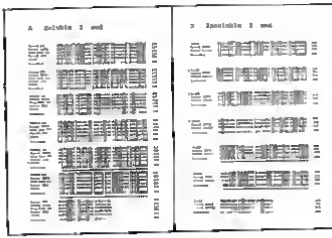

A Soluble A end
A Insoluble A end

Figure 3-7 DNA gel blot analysis of A, Approximate size of the *Ivr1*, *Ivr2*, *Ivr2C-1* and *Ivr2C-2* cDNA clones and B Extent of cross-reactivity between them A, EtBr stained DNA gel blot analysis of approximate length for *Ivr1*, *Ivr2*, *Ivr2C-1* and *Ivr2C-2* B and C, DNA gel blots with equal amounts (1 µg) of recombinant DNA from each cDNA (Released from pUC 19 vector by digestion with EcoRI) and probed with ^{32}P labeled fragments from either A, *Ivr1* (Kpn I-EcoR I 1 kb fragment) or B *Ivr2* (Pst I-Pst I 200 bp fragment) Both blots were exposed to X-ray film for 1 hr Sites on the restriction maps are K, Kpn I, P, Pst I R, EcoR I

A
MW
Tvr1
Tvr2
Tvr2C-1
Tvr2C-2
2.2
1.4
1.2
B
probed with Tvr1
K
R
Tvr1
Tvr2
Tvr2C-1
Tvr2C-2
1.2
C
probed with Tvr2
P P
Tvr1
Tvr2
Tvr2C-1
Tvr2C-2
2.2
1.4
1.2

Discussion

Soluble invertase genes were cloned and characterized for two reasons. The first of these was to characterize the extent of their carbohydrate-responsiveness relative to that of genes for sucrose synthase, ultimately to provide a more complete picture of how sugars influence the capacity for their own metabolism at the transcriptional level. The second planned use for the invertase clones was to clarify the potential significance of these gene family members during development and/or environmental adjustment by maize tissue and organs.

Three lines of evidence support the designation of these genes not only as maize invertases but also as soluble ones. First, the full length sequence of the putative maize invertase clone (*Ivr1G*) has extensive sequence similarity to other invertases found in vascular plants (Table 3-1), and shares the conserved key domains identified in other invertases (NDPNG [β-fructosidase motif, Sturm and Chrispeels, 1990] plus FRDPTTA, TGMWEC and YASKTF) (Figure 3-5). Second, the maize invertase gene examined here has a considerably greater amino acid identity to the soluble isoforms of invertase than to the cell wall-bound ones found in other vascular plants (Table 3-1, Figure 3-6). The underlined areas in figure 3.6 are those highly conserved regions which are shared among soluble invertases but not insoluble ones. In particular, the amino acid sequence at the C-terminus of the *Ivr1G* protein is significantly more similar to that of soluble vs. insoluble forms

Targeting signals for vacuolar proteins are frequently present in this region as C-terminal propeptides (Bednarek et al., 1990, Chrispeels, 1991, Bednarek and Raikhel 1992). Third, message abundance of *Ivr1* and *Ivr2* correlates well with total soluble invertase activities in an array of maize tissues and/or developmental stages (see Chapter 4).

Invertases of maize and other vascular plants are presumably encoded by different genes, although in yeast, variable splicing allows a single gene to encode both cell-wall-bound and soluble invertases (Carlson and Botstein, 1982). There are at least two *Ivr1* like genes in the maize genome, and the *Ivr1* and *Ivr2* subfamilies have been tentatively mapped to two and four different loci respectively (data not shown, collaboration with Scott Wright, Genetic Linkages, Salt Lake, Utah).

The genomic clone of maize invertase has typical CAAT and TATA boxes located in the upstream untranslated region (Figure 3-2). The second exon is unusually small (9 bp) in maize *Ivr1* invertase (Figure 3-2) and tomato soluble invertase genes (Elliott et al. 1993). The amino acids encoded by this 9 bp exon are located in a highly conserved domain found in all invertase clones (NDPNG, the β-fructosidase motif, Sturm and Chrispeels, 1990). This represents one of the smallest exons currently known to function in the plant genome (M. Schuler, personal communication).

In the *Ivr1* maize invertase genomic gene, several introns (number 1, 3, 4 and 5) are also found to contain one or more copies of an RY sequence motif (CATGCATG, data not shown), which thus far has been implicated in seed-specific

gene expression (Dickinson et al., 1988; Baumlein et al., 1992; Lelievre et al., 1992). This suggestion is also supported by the preferential expression of the *Ivr1* subfamily genes in reproductive tissues (see Chapter 4).

The polypeptide encoded by the *Ivr1G* invertase gene has 670 residues with a molecular weight of 71,942 (Figure 3.3). The calculated isoelectric point is 7.5, which is intermediate between that of carrot soluble invertases (SI: 3.8, SII: 5.7, Unger et al., 1994) and insoluble invertases (carrot CW: 9.9, Sturm and Chrispeels, 1990; *Arabidopsis* CW: 9.1; Schwebel-Dugue et al., 1994). This protein also contains five putative glycosylation sites (N-X-S/T) and a potential peptide signal from M^1 to A^{22} (Figure 3-3).

CHAPTER 4
DEVELOPMENTAL EXPRESSION AND CARBOHYDRATE RESPONSIVENESS OF TWO MAIZE INVERTASE GENE SUBFAMILIES

Introduction

Invertases (β-fructosidase, EC 3.2.1.26) play a key role in sugar metabolism. In vascular plants, different isoforms are located in different cellular compartments (Avigad, 1982; Hawker, 1985). Isoforms with an acidic pH optimum are found in the vacuole and/or apoplasm whereas isoforms with a neutral pH optimum are located in the cytoplasm. Work with sugar cane stems (Hawker and Hatch, 1965; Glasziou and Gayler, 1972) and corn kernels (Shannon, 1968; Shannon, 1972; Shannon and Doughty, 1972; Shannon et al, 1993) has indicated that imported sucrose moves from phloem into the extracellular space where it is hydrolyzed by a cell-wall bound, acid invertase. This is presumed to contribute to a sucrose concentration gradient between the phloem and apoplast, facilitating transfer of sucrose into importing tissues (Lin et al., 1984; Doehlert, 1986; Doehlert and Felker, 1987; Doehlert et al., 1988; Tugeon, 1989). Soluble invertase has been found in the vacuoles of sucrose-storing cells (Avigad, 1982). Thus, soluble invertases with acidic pH optima are often thought to be localized in the cell vacuoles of other tissues as well, where they can mobilize sucrose temporarily stored in this compartment.

Rapidly expanding tissues require either invertase or sucrose synthase to convert sucrose into substrates necessary for respiratory and synthetic processes (Giaquinta, 1979, Avigad, 1982, Morris and Arthur, 1984b, Hawker 1985, Schaffer et al., 1987). Invertase can be especially important to cell expansion through generation of hexoses and their associated osmotic potential (Kaufman, 1973 Avigad, 1982 Schmalstig and Cosgrove 1988 1990). Invertase activity is also associated with reproductive organs (Jaynes and Nelson, 1971a, Shannon and Dougherty 1972 Singh and Knox, 1984 Hubbard et al., 1989, Miron and Schaffer, 1991, Klann et al 1992, Reger et al 1992). Invertase can aid competition for sink capacity for reproductive growth. Soluble invertase is the predominant enzyme for sucrose breakdown during the early developmental stage of maize kernel (Tsai et al., 1970) and snap bean seed (Sung et al., 1994).

Genes regulated by carbohydrate were first studied in microorganisms (Carlson, 1987 Schuster 1989). Those genes are usually involved in metabolic pathways. In vascular plants, sugar responsive genes have been primarily characterized in storage organs (Rocha-Sosa et al 1989, Salanoubat and Belliard 1989, Muller-Rober et al., 1990 DeWald et al 1994, Sadka et al., 1994). Those genes generally encode storage proteins such as patatin from potato (Rocha-Sosa et al., 1989) tuberous root storage protein genes from sweet potato (Hattori et al., 1990) vegetative storage proteins from soybean (Sadka et al., 1994). In addition, carbohydrate-induced changes in gene expression have also focused on metabolic pathways, especially those involved in sugar metabolism such as sucrose synthase

(Maas et al., 1990, Koch et al., 1992), malate synthase, isocitrate lyase (Graham et al. 1994) and/or photosynthetic pathway (Sheen, 1990) and are considered critical mechanisms for sensing environmental and developmental signals.

Invertase is one of the only two enzymes known for sucrose breakdown in vascular plants and has shown a relatively long term responsiveness to carbohydrate availability at the enzyme level (Sacher et al., 1963, Glasziou et al., 1966, Ricardo et al., 1972, Kaufman et al., 1973).

Previous research indicated that invertase was vital at both the specific organ level and at the whole plant level. Robbins (1958) found that OH43 primary roots could not grow on sucrose agar, and Duke et al. (1991) showed these roots to be invertase deficient. Miller and Chourey (1992) also found that the abnormal development of *miniature* kernels was associated with an invertase deficiency. The present study utilizes two acid invertase gene-probes to determine the effects of developmental processes and altered carbohydrate availability on expression of the *Ivr1* and *Ivr2* classes for soluble acid invertase genes. The report presented here also demonstrates the extent of developmental differences and carbohydrate responsiveness in two subfamilies of maize genes for acid invertase (probably soluble). These findings indicate that there may be specific roles for soluble invertases during development and that these could differentially contribute to adjustment of sucrose import, cellular volume, and possibly metabolism in vascular plants.

Materials and Methods

Plant Material

The *Zea mays* hybrid NK 508 was used for all experiments. For analyses of developmental changes, plants were grown under greenhouse or field conditions. Samples harvested included leaves, anthers, silk, cobs, pollen, prop roots, and kernels at different developmental stages.

For experiments with root tips, seeds were first smersed in 20% Clorox for 30 min, followed by 30 min of continuous rinsing with water. Germination took place in the dark at 18 °C on two layers of moist 3 MM paper (Whatman, Inc., Clifton, NJ) in 17 x 26 cm glass pans. Air flowed continuously at 1 liter min^{-1} through each pan for the 6-day period, with 40% O_2 supplied during the final 24 hr before root tip excision. The moisture level was adjusted daily by applying mist and draining excess water. The terminal 1 cm was cut from root tips (of ca. 3 to 6 cm total length) under a sterile transfer hood.

Experimental Conditions

Experimental treatments were as described by Koch et al. (1992). Approximately 100 root tips (~ 500 mg) were used for each experimental treatment. Excised root tips were incubated in the dark at 18 °C for 6 to 48 hr in Whites'

medium, either with or without an array of supplemental sugars Each group of root tips was agitated at 120 cycles per minute in a 125 ml side-arm Erlenmeyer flask with 50 ml of sterile media. Airflow (40% O_2, make sure to supply enough O_2) through air stones in each flask was maintained at 250 ml min^{-1} throughout the incubations

RNA Isolation and Gel Blot Analysis

Root tip samples were rinsed twice in sterile water, blotted dry weighed, and frozen in liquid N_2 Other samples (as mentioned in the previous text) were harvested from greenhouse and/or field grown plants, weighed, and frozen immediately in liquid N_2 Samples were ground into fine power in liquid N_2 and total RNA was extracted (McCarty 1986) RNA was quantified spectrophotometrically (Sambrook et al , 1989) Total RNA (10 µg) was separated by electrophoresis in 1 % agarose gels containing formaldehyde (Thomas 1980) blotted to nylon membranes, and fixed by baking and/or UV treatment (Sambrook et al , 1989) Filters were hybridized at 65 °C in a solution with 7 % SDS 250 mM Na_2HPO_4 (pH 7 2) and 1 % BSA (Church and Gilbert, 1984) Maize *Ivr 1* and *Ivr 2* invertase cDNA clones were radiolabeled by random primer No cross-reactivity was observed between the *Ivr 1* and *Ivr 2* gene probes when hybridizations were conducted at high stringency (data not shown). Blots were washed as described by

Church and Gilbert (1984) and exposed against X-ray film with intensifying screens at -80 °C.

Enzyme Extraction

Soluble invertase was extracted as per Duke et al. (1991). Frozen tissue samples were ground to a fine powder in liquid N_2 using a mortar and pestle. Frozen powder was transferred to a second mortar containing ice-cold 200 mM HEPES buffer (pH 7.5) with 1 mM DTT, 5mM $MgCl_2$, 1 mM EGTA and 10%(w/w) PVPP. One ml of extraction buffer was used for each 100 mg of tissue fresh weight. Buffered extract was centrifuged at 15,000 x g for 10 min to sediment particulate matter. Pellets were saved for salt-solubilized particulate invertase extraction. Supernatant was dialyzed (50 000 MW cutoff) at 4 °C for 24 hr against extraction buffer diluted 1:40. Buffer was changed twice. Soluble dialyzed extract was centrifuged again at 15 000 x g for 10 min and supernatant assayed for soluble invertase activity as described below.

Insoluble invertase was extracted as described by Dochlert and Felker (1987). Pellets remaining from extractions of soluble invertase were washed three times by sequentially resuspending each in 5 to 10 ml extraction buffer and centrifuging at 15 000 x g for 10 min. Salt-solubilized particulate invertase was extracted by resuspending the pellet in extraction buffer containing 1 M NaCl. Solubilized particulate invertase was recovered in supernatant following centrifugation at 15 000

x g for 10 min. Pooled supernatant fractions were assayed for insoluble invertase assay as described below

Enzyme Assay

Both soluble and salt-solubilized invertase activities were assayed for 15 to 30 min at 37 °C in an assay medium with 100 mM Na-acetate (pH 4.5) and 100 mM sucrose in a final volume of 500 µl. Activity was determined by measuring reducing sugars as described by Nelson (1944) and Somogyi (1951)

Results

Developmental and organ-level differences were evident in expression of the two classes of invertase genes (Figure 4-1 A). Message levels for the *Ivr1* group were markedly higher in reproductive structures than vegetative tissues, whereas those of the *Ivr2* type transcripts were abundant in essentially all of the sucrose-importing structures examined (loading same amount of total RNA). Message from both classes of invertase were present in sink leaves, dropping below detectable levels during sink-to-source transition. Transcript levels of both types were also evident in those tissues undergoing rapid growth and/or cell division, such as root tips, anthers, pollen and silk (styles). As observed for relative mRNA abundance, activity of this enzyme fraction also predominated in the most rapidly elongating tissues (such as root tips and silk) regardless of whether data were expressed per unit protein or fresh weight (data not shown). Activity was also generally elevated in instances of enhanced sucrose import. The greater ratio of RNA/protein recovered from root tip extracts vs those from other tissues suggests that if changes in total RNA encoding invertase messages are viewed relative to protein levels, then invertase mRNA levels in root tips are greater relative to enzyme activity than is evident in Figure 4-1. The greater values for RNA/protein recovery from root tip extracts may possibly be due to the extensive meristematic activity in these organs.

Shifts in region of localization were evident during kernel development for the two subfamilies of maize invertase (Figure 4-2 A and Figure 4-3 A). Message

(Days After Pollination) dropping below detection within 16 DAP However the *Ivr2* type mRNA was abundant in the pedicel region and barely detectable in the middle and top portions of the kernels (Figure 4-3A) In contrast, levels of *Ivr1*-related messages in the pedicel region were similar or less than those in the middle and top sections of kernels at the same developmental stage In addition developmental differences in timing were evident, with a narrow peak in *Ivr1* transcript abundance at 8 DAP in the upper kernel, vs a broader elevation in *Ivr2* message in the pedicel between 8 and 12 days after pollination. Transcript levels of the *Ivr1* subgroup were approximately similar in pericarp and endosperm at 10 DAP, whereas the *Ivr2* mRNAs were considerably more abundant in the pericarp (data not shown)

Figure 4-3 B showed that total soluble acid invertase activity, like that of *Ivr1* and *Ivr2* mRNA was highest in the pedicel region and lowest in the top area of the same kernels when expressed per unit total soluble protein (similar results were observed when data were calculated per unit fresh weight [data not shown] except that peak activity was elevated for two days longer) Total activity of soluble acid invertase was maximal in extracts of kernels sampled 12 days after pollination, dropping gradually to below detection in those from between 20 and 24 DAP (Figure 4-2C) In contrast, salt solubilized particulate invertase activity (insoluble) increased gradually in developing kernels but did so most rapidly between 2 and 6 DAP Peak activity was observed at ca 16 DAP and decreased slowly thereafter Activity remained detectable at 32 DAP (well past maturity under local growing

Activity remained detectable at 32 DAP (well past maturity under local growing conditions). This salt-solubilized activity was also maximal in the pedicel area and lowest in the top portion of the same kernels when expressed per unit total salt-solubilized protein. If decreases in mRNA levels encoding *Ivr1* and *Ivr2* are viewed relative to protein levels, then the drop in message abundance is more pronounced than pictured due to the onset of enhanced protein storage in kernels between 10 and 12 DAP.

During the earliest stages of kernel development, message levels for both *Ivr1* and *Ivr2* subfamilies and total soluble invertase activity increased markedly (Figure 4-4). Soluble invertase activity from kernels two days after pollination was twice as high as that of unpollinated ones (Figure 4-4B) and insoluble activity from the same kernels (Figure 4-4C).

During anther development, transcript levels of both *Ivr1* and *Ivr2* classes of invertase increased gradually through anthesis (Figure 4-5A). Both message types were also abundant in RNA extracted from pollen. This was probably not the basis for localization in young anthers, because shedding anthers had greater apparent levels of both classes of mRNA than did pollen itself.

Both the *Ivr1* and *Ivr2* types of mRNA were abundant in silk if tissue was sampled before or immediately after pollination (Figure 4-6A). A gradient in relative message levels for these gene classes was also evident along the length of the silk, with lowest levels in the top (distal) 1/3 and greatest abundance in the 1/3 closest to the ovary (proximal) (Figure 4-7A). A rapid response to pollination was

also observed in a progressive decline of message levels for both classes of invertase transcripts. The longitudinal gradient of invertase mRNA levels from tip to base of silks was reduced during this decrease by the rapid decline in message abundance observed in the basal region of the style. At the enzyme level temporal and spatial changes in total soluble activity were consistent with those of the *Ivr1* and *Ivr2* message levels (Figure 4-6B, 4-7B). Salt-solubilized invertase was relatively constant before and/or after pollination, and no activity gradation was evident along the length of silk (Figure 4-6C, 4-7C). The drop in mRNA abundance of invertase is still more pronounced than pictured if considered relative to protein levels.

Differential responses of the *Ivr1* and *Ivr2* class genes to sugar supplies became apparent when excised root tips were supplemented with a range of glucose and/or sucrose concentrations and incubated for 24 hr (Figure 4-8). *Ivr1* class message levels were maximal with ca 0.5% exogenous glucose (Figure 4-8A) and ca 0.2% sucrose (Figure 4-8B), whereas those of *Ivr2* remained relatively constant when media glucose and/or sucrose levels were between 0.2 and 4.0%. In addition, levels of the *Ivr1* subfamily of transcripts appeared to drop less markedly during a 24 hr period without exogenous carbohydrate than did those of *Ivr2* (Figure 4-8). In excised maize root tips, soluble sugars reportedly drop to minimal levels within 10 hr if no supplemental sugars are provided (Sagho and Pradet, 1980). Differential responses of the *Ivr1* and *Ivr2* classes of invertase to carbohydrate deprivation were further explored by an analysis of their progression over time in excised root tips (Figure 4-9). Levels of the *Ivr1* type mRNAs decreased less rapidly than did those

of the *hvr2* subgroup and persisted for considerably longer Relatively little change was evident during 24 hr of starvation and message remained readily apparent for at least 48 hr In contrast, levels of the *hvr2* class of mRNA dropped below detection after between 12 and 18 hr of carbohydrate deprivation (Figure 4-9)

Although *hvr1* message abundance appeared to be relatively insensitive to an 18-hr starvation period or subsequent additions of sugar to media, levels of mRNA for the *hvr2* subfamily were sensitive to both (Figure 4-10) Glucose replacement after 18 hr of C-depravation appeared to counter initial decreases in levels of message for the *hvr2* subfamily These returned to pre-starvation levels after 15 hr incubation in 0 5 % glucose (Figure 4-10)

The responses of the *hvr1* and *hvr2* class genes to different types of sugars (Figure 4 11A) also showed that expression of both appeared to require a supply of metabolizable sugars Transcripts remained abundant in the presence of 2% D-glucose fructose, or sucrose in the exogenous media, but dropped when these were replaced by either L-glucose or mannitol

Figure 4-1 Abundance of mRNA from the *Ivr1* and *Ivr2* subfamilies of soluble acid invertase and activity of total soluble and insoluble maize invertases in root tips, a sink leaf, a source leaf, a prop root, anthers, silk and kernels (2 DAP). A. RNA gel blots with equal amounts (10 μg) of total RNA from above tissues were probed with ^{32}P-labeled *Ivr1* or *Ivr2* representing the two subfamilies of maize soluble acid invertase. Blots were exposed to X-ray film for 24 or 12 hr, respectively. B. Total soluble acid invertase activity from the above tissues. C. Insoluble acid invertase activity from the above tissues. Values for RNA/protein recovery were ca. 0.04 (± 0.02) for tissues other than root tips and did not otherwise differ significantly between tissue types. Root tip values were greater (0.15 ± 0.04) possibly due to more extensive meristematic activity.

A. mRNA
root tips
sink leaf
source leaf
prop root
anthers
silk
kernel (20AP)
Probe
Ivr1
Ivr2
B Soluble activity
Invertase activity
(μmol glucose mg protein hr⁻¹)
60
40
20
0
C. Insoluble activity
20
0
root tips
sink leaf
source leaf
prop root
anthers
silk
kernel (20AP)

Figure 4-2. Abundance of mRNA from the *Ivr1* and *Ivr2* subfamilies of soluble acid invertase and activity of total soluble and insoluble maize invertases during kernel development. A and B, RNA gel blots with equal amounts (10 μg) of total RNA from kernels between 6 and 32 DAP (full maturity at ca 30 DAP under local conditions) were probed with ^{32}P labeled *Ivr1* or *Ivr2* representing the two subfamilies of maize soluble acid invertase. Blots were exposed to X-ray film for two days. Relative abundance of mRNA was quantified by phosphor image quantifications. C. Total soluble acid invertase activity from the above tissues. D. Insoluble acid invertase activity from the above tissues. Values for RNA/protein recovery from this set of kernels were ca. 0.04 (±0.02), except at 10 and 12 DAP (0.08 ± 0.04) (consistent with changes in cell division and protein levels during early kernel development).

A. Ivr1 mRNA
B. Ivr2 mRNA
C. Soluble activity
D. Insoluble activity
% of mature response
Invertase activity
(μmol glucose mg⁻¹ protein hr⁻¹)
DAP

Figure 4-3 Abundance of mRNA from the *Ivr1* and *Ivr2* subfamilies of soluble acid invertase and activity of total soluble and insoluble maize invertases in pedicel, middle and top portions of kernels at 8, 10 and 12 DAP. A, RNA gel blots with equal amounts (10 μg) of total RNA from above tissues were probed with ^{32}P-labeled *Ivr1* or *Ivr2* representing the two subfamilies of maize soluble acid invertase. Blots were exposed to X-ray film for two days. B. Total soluble acid invertase activity from the above tissues. C, Insoluble acid invertase activity from the above tissues. Values for RNA/protein recovery were ca. 0.03 (± 0.01) with variability independent of tissue gradient from kernel top to pedicel. Values for RNA/protein recovery dropped from ca. 0.04 (± 0.02) to 0.02 (± 0.01) past 10 DAP, and is consistent with elevated protein storage in kernels at this stage.

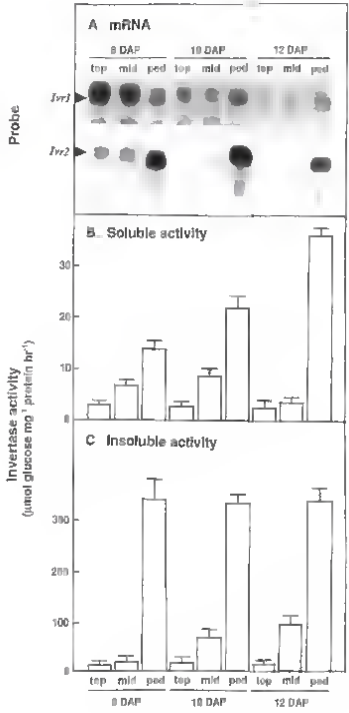

A mRNA
8 DAP
10 DAP
12 DAP
top mid pod top mid pod top mid pod
Probe
Ivr1
Ivr2
B Soluble activity
C Insoluble activity
Invertase activity
(μmol glucose mg⁻¹ protein hr⁻¹)
30
20
10
0
300
200
100
0
top mid pod top mid pod top mid pod
8 DAP
10 DAP
12 DAP

Figure 4-4 Abundance of mRNA from the *Ivr1* and *Ivr2* subfamilies of soluble acid invertase and activity of total soluble and insoluble maize invertases in kernels (ovules) sampled daily from 2 days before to 2 days after pollination A RNA gel blots with equal amounts (10 μg) of total RNA from above tissues were probed with ^{32}P labeled *Ivr1* or *Ivr2* representing the two subfamilies of maize soluble acid invertase Blots were exposed to X-ray film for 24 or 12 hr respectively B Total soluble acid invertase activity from the above tissues C Insoluble acid invertase activity from the above tissues Values for RNA/protein recovery were ca 0 03 (± 0 01) with variability independent of development

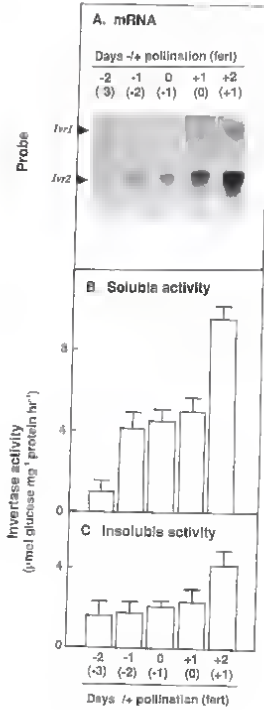

A. mRNA
Days -/+ pollination (fert)
-2 (-3)
-1 (-2)
0 (-1)
+1 (0)
+2 (+1)
Probe
Ivr1
Ivr2
B. Soluble activity
C. Insoluble activity
Invertase activity
(μmol glucose mg⁻¹ protein hr⁻¹)
8
4
0
Days -/+ pollination (fert)

Figure 4-5 Abundance of mRNA from the *Ivr1* and *Ivr2* subfamilies of soluble acid invertase and activity of total soluble and insoluble maize invertases during the final 3 days of anther development and in mature pollen. A. RNA gel blots with equal amounts (10 µg) of total RNA from above tissues were probed with ^{32}P-labeled *Ivr1* or *Ivr2* representing the two subfamilies of maize soluble acid invertase. Blots were exposed to X-ray film for 24 or 12 hr respectively. B. Total soluble acid invertase activity from the above tissues (* not assayed). C. Insoluble acid invertase activity from the above tissues (* not assayed). Values for RNA/protein recovery were ca. 0.03 (±0.01) for mature and shedding anthers. Values from extracts of young anthers were greater (0.07 ± 0.03) possibly due to more extensive meristematic activity.

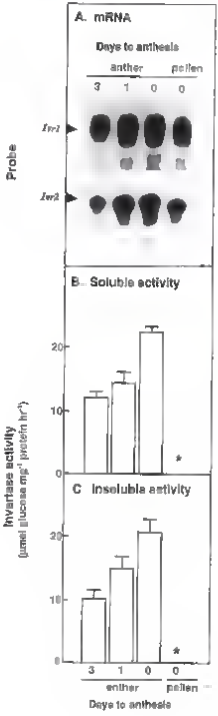

A. mRNA
Days to anthesis
anther
pollen
3
1
0
0
Probe
Ivr1
Ivr2
B. Soluble activity
C. Insoluble activity
Invertase activity
(μmol glucose mg⁻¹ protein hr⁻¹)
20
10
0
*
3
1
0
0
anther
pollen
Days to anthesis

Figure 4-6 Abundance of mRNA from the *Ivr1* and *Ivr2* subfamilies of soluble acid invertase and activity of total soluble and insoluble maize invertases in silk sampled daily from 2 days before to 2 days after pollination. A, RNA gel blots with equal amounts (10 μg) of total RNA from above tissues were probed with ^{32}P-labeled *Ivr1* or *Ivr2* representing the two subfamilies of maize soluble acid invertase. Blots were exposed to X-ray film for 24 or 12 hr, respectively. B, Total soluble acid invertase activity from the above tissues. C, Insoluble acid invertase activity from the above tissues. Values for RNA/protein recovery were ca. 0.06 (± 0.02) before pollination and dropped to 0.02 (± 0.01) after pollination. Transcription may be markedly reduced by pollination and/or message longevity may largely determine the extent of change in types of mRNA predominating.

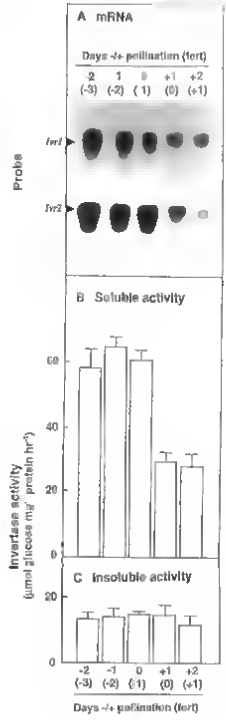

A mRNA
Days -/+ pollination (fert)
-2 (-3)
-1 (-2)
0 (1)
+1 (0)
+2 (+1)
Probe
Ivr1
Ivr2
B Soluble activity
C Insoluble activity
Invertase activity (μmol glucose mg⁻¹ protein hr⁻¹)
0
20
40
60
-2 (-3)
-1 (-2)
0 (1)
+1 (0)
+2 (+1)
Days -/+ pollination (fert)

Figure 4-7 Abundance of mRNA from the *Ivr1* and *Ivr2* subfamilies of soluble acid invertase and activity of total soluble and insoluble maize invertases in tip, mid and low portions of silk (portions of silk [ca 4 cm total length] relative to ovary) sampled at pollination 3 hr later 6 hr later or after 24 hr A RNA gel blots with equal amounts (10 µg) of total RNA from above tissues were probed with ^{32}P-labeled *Ivr1* or *Ivr2* representing the two subfamilies of maize soluble acid invertase. Blots were exposed to X ray film for 24 or 12 hr respectively B Total soluble acid invertase activity from the above tissues C Insoluble acid invertase activity from the above tissues. Values for RNA/protein recovery were ca 0 06 (± 0 02) before pollination and dropped to 0 04 (± 0 02) within 3hr after pollination then to 0 02 (± 0 01) at 6hr and 24hr after pollination Transcription may be markedly reduced by pollination and/or message longevity may largely determine the extent of change in types of mRNA predominating

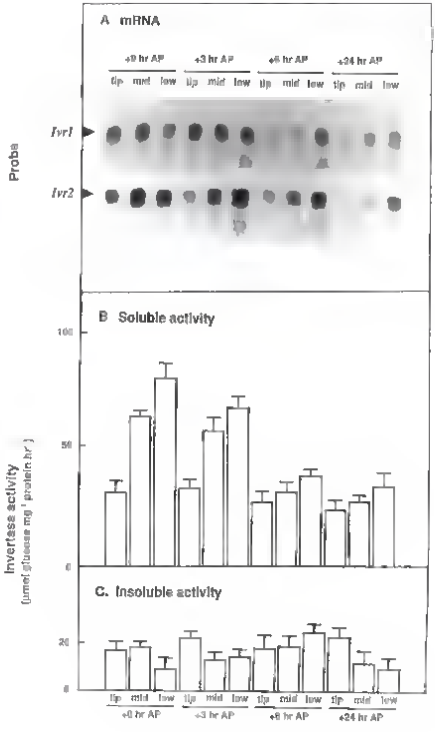

A mRNA
+0 hr AP
+3 hr AP
+8 hr AP
+24 hr AP
tip mid low
Probe
Ivr1
Ivr2
B Soluble activity
100
50
0
C. Insoluble activity
20
0
Invertase activity
(umol glucose mg-1 protein hr-1)

Figure 4-8 Abundance of mRNA from the *Ivr1* and *Ivr2* subfamilies of soluble acid invertase in maize root tips incubated for 24 hr in White s basal salts medium supplemented with either 0 0 2 0 5 2 0 4 0% glucose or sucrose RNA gel blots with equal amounts (10 μg) of total RNA from above tissues were probed with ^{32}P-labeled *Ivr1* or *Ivr2* representing the two subfamilies of maize soluble acid invertase Blots were exposed to X-ray film for 24 or 12 hr respectively

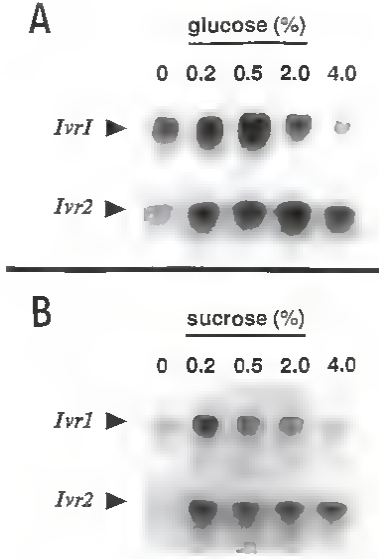

A
glucose (%)
0 0.2 0.5 2.0 4.0
Ivr1
Ivr2
B
sucrose (%)
0 0.2 0.5 2.0 4.0
Ivr1
Ivr2

Figure 4-9 Abundance of mRNA from the *Ivr1* and *Ivr2* subfamilies of soluble acid invertase in maize root tips depleted of carbohydrates for either 6, 12, 18, 24, 36, or 48 hr respectively, in White's basal salts medium without an exogenous sugar supply. RNA gel blots with equal amounts (10 µg) of total RNA from above tissues were probed with ^{32}P-labeled *Ivr1* or *Ivr2* representing the two subfamilies of maize soluble acid invertase. Blots were exposed to X-ray film for 24 or 12 hr, respectively.

starvation (hr)
6 12 18 24 36 48
Ivr1
Ivr2

Figure 4-10 Abundance of mRNA from the *Ivr1* and *Ivr2* subfamilies of soluble acid invertase during post-starvation recovery of maize root tips. Sugar depletion in White s basal salts without sugars (18 hr) was followed by incubation for various periods of time (6-18 hr) in media with 0 5% glucose supplements RNA gel blots with equal amounts (10 μg) of total RNA from above tissues were probed with ^{32}P-labeled *Ivr1* or *Ivr2* representing the two subfamilies of maize soluble acid invertase Blots were exposed to X-ray film for 24 or 12 hr respectively

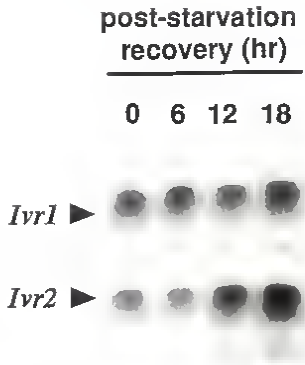
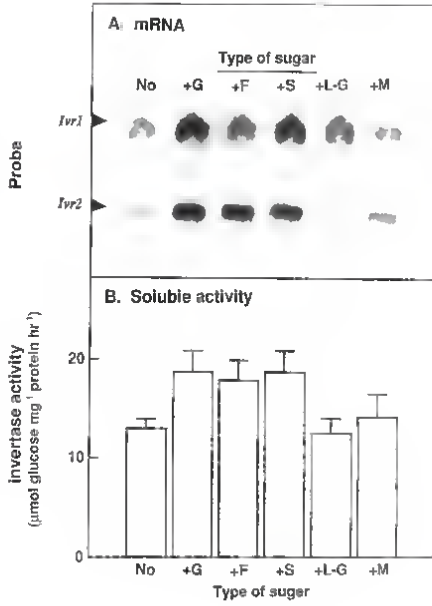

post-starvation
recovery (hr)
0 6 12 18
Ivr1
Ivr2

Figure 4-11 Abundance of mRNA from the *Ivr1* and *Ivr2* subfamilies of soluble acid invertase and activity of total soluble acid invertase in maize root tips incubated for 24 hr in White's basal salts medium supplemented with either 2.0% glucose, fructose, sucrose, L-glucose or mannitol respectively. A. RNA gel blots with equal amounts (10 μg) of total RNA from above tissues were probed with ^{32}P labeled *Ivr 1* or *Ivr 2* representing the two subfamilies of maize soluble acid invertase. Blots were exposed to X-ray film for 24 or 12 hr. respectively. B. Total soluble acid invertase activity from the above samples. Insoluble invertase activity (not shown) was consistently ca. 10-fold less than that in the soluble fraction of maize root tips. Values for RNA/protein recovery were ca. 0.14 (± 0.05) with variability independent of presence or absence of metabolizable C-source.


A. mRNA
Type of sugar
No
+G
+F
+S
+L-G
+M
Probe
Ivr1
Ivr2
B. Soluble activity
invertase activity
(μmol glucose mg⁻¹ protein hr⁻¹)
20
10
0
No
+G
+F
+S
+L-G
+M
Type of suger

Discussion

The significance of findings presented here extends from implications of special roles for soluble invertases during development (especially pollination and early kernel development) to broader possible contributions to adjustment of sucrose import, cell volume, and metabolism in a multi-celled higher plant. The spatial and temporal patterns of expression for the two invertase subfamilies, as well as the contrast between them suggest involvement in specific developmental processes. The availability of these clones has also allowed the hypothesis to be tested that regulation of transcript level by photosynthate availability could contribute to adjustment of both avenues for sucrose breakdown in a cell (invertase as well as sucrose synthase). Moreover, a surprising similarity in differential carbohydrate responsiveness was evident between the two invertase subfamilies and the two sucrose synthase genes. In both instances, the more broadly distributed of the two (*Ivr2* or *Sus1*) was found to be readily induced by enhanced carbohydrate availability, whereas the form which was upregulated during more specific developmental and environmental signals (*Ivr1* or *Sh1*) was less sensitive to sugar supplies (Koch et al., 1992).

The present work indicates that each subfamily of the invertase genes is expressed differentially depending on developmental stage and the tissue/organ involved. Although invertase activity was detected in extracts of almost every sucrose importing tissue examined, the *Ivr1* type message was preferentially

associated with reproductive organs (Figure 4-1 A). Data shown here for general association between soluble invertase activity and rapid growth/cell division were consistent with previous suggestions for the role of this enzyme relative to sucrose import. Rapidly expanding tissues require either invertase or sucrose synthase to convert sucrose to substrates necessary for respiratory and synthetic processes (Glaszmou and Gayler, 1972, Giaquinta, 1979, Avigad 1982, Morris and Arthur, 1984b, Hawker 1985, Schaffer, 1986, Schaffer et al. 1987). Invertase in particular can be important to cell expansion through generation of hexoses and their associated osmotic potential. Changes in both message and activity in the present study were also consistent with the gradual sink-to-source transition in leaves (Ho, 1988, Turgeon, 1989, Nguyen-Quoc et al., 1990).

Data presented here indicate that soluble invertases may be especially important during the early stages of maize kernel development. This is consistent with a hypothesis advanced on the basis of previous work (Hanft and Jones, 1986a, 1986b, Reed and Singletary, 1989), which suggests that the soluble forms of these enzymes in the pedicel may be critical to initiation of normal kernel development. The expression pattern of both the *Ivr1* and *Ivr2* classes of invertase, as well as total soluble activity (Figure 4-2 and Figure 4-4), are also in agreement with this possibility.

Past research on invertase and kernel development has tended to focus on the insoluble "cell-wall-bound" form of this enzyme primarily because of its apparent importance during later stages of kernel fill. Shannon and coworkers proposed that

the driving force for assimilate movement into normally developing kernels was the sucrose gradient between the leaves and the pedicel apoplasm combined with the monosaccharide-gradient between the pedicel apoplasm and the starchy endosperm cells (Shannon, 1968, Shannon, 1972, Shannon and Dougherty 1972) Both gradients are presumably maintained by the activity of an apoplastic pedicel invertase (Shannon et al 1993)

Early phases of kernel growth may differ from previous hypothesis, despite the apparent importance of insoluble invertase and sucrose synthase activity in later development (ca 22 DAP) (Tsai et al 1970 Chourey and Nelson, 1976, Chourey 1981) Sucrose synthase activity is not detectable prior to 12 DAP (Tsai et al.,1970, Chourey and Nelson 1976 Chourey, 1981) Instead, activity of soluble invertase as well as mRNA for both classes of soluble invertase peak at 8 to 12 DAP (Tsai et al , 1970, Figure 4-2) Soluble acid invertase may also be important to initial establishment and maintenance of sink strength

The potential role of soluble invertase genes in early kernel development may be related to the difference between tissues in which message classes were most strongly expressed *Ivr1* mRNA levels were greater in endosperm and the upper kernel whereas *Ivr2* message was most abundant in the pedicel region (Figure 4-3A) This distinction could be important for two reasons The first of these is the suggestion that the *miniature* phenotype results initially from reduced endosperm invertase and its subsequent effect on pedicel invertase (both presumably insoluble Miller and Chourey, 1992) The second is the hypothesis advanced by Hanft and

Jones (1986a; 1986b) which tentatively attributes kernel abortion under water and heat stresses to reduced activity of soluble invertase in the pedicel. The following scenario represents one possible explanation for the combination of data on the greater sensitivity of the *Ivr2* genes to carbohydrate deprivation and the abundance of their transcripts in the pedicel. Any early limitation to assimilate flux into the endosperm would be expected to reduce soluble sugar concentration in the pedicel within a relatively short time (Hanft and Jones, 1986a). The depletion of pedicel sugars could in turn result in decreased levels of the carbohydrate-responsive *Ivr2* gene products and a subsequent decrease in soluble invertase activity in this region. This is consistent with the observation that it is the soluble rather than insoluble acid invertase activity which is most markedly affected in pedicel of kernels that have been induced to abort vs nonaborting kernels (Hanft and Jones, 1986a).

The role of soluble invertases during anther and pollen development is probably twofold. First, there are no plasmodesmatal connections between developing pollen grains and the surrounding tapetum layer (Kesselbach, 1949). The tapetum thus lies at the terminal end of the maternal transport path. Any invertase or sucrose synthase present in these cells could theoretically enhance sugar transport to pollen grains by creating a sucrose gradient between phloem and the secretory surface, much as hypothesized for developing kernels (Shannon 1972, Shannon and Dougherty, 1972; Lin et al., 1984). Presumably, enhanced hydrolysis could also benefit the probable elevation in respiratory and biosynthetic demands. Our results

indicated that significant amounts of both *Ivr1* and *Ivr2* type message were present in the anther tissues collectively (Figure 4-5)

Second, invertase message in pollen may be important for subsequent germination to facilitate use of endogenous as well as exogenous sucrose Sucrose represents the major soluble sugar present in the majority of angiosperm pollen grains, including maize. Mature pollen grains from diverse plants contain sucrose but not starch as reserve carbohydrate (Pottnot and Horovitz, 1977; Nakamura et al., 1980) Germinating pollen grains show an extremely high rate of growth and thus have a high demand for carbon skeletons required for pollen tube wall synthesis as well as substrates for respiration (Hoekstra, 1983 Singh and Knox 1984) The sucrose content from pollen grains of *Camellia japonica* decreases rapidly during pollen growth and the activity of soluble invertase increases during culturing and a high constant activity is found at the later stages of pollen tube growth (Nakamura et al., 1980) Our data are consistent with the possibility that invertase has multiple functions during anther development and pollen grain maturation (Figure 4-5) Further localization of invertase at the message and/or protein level *in situ* could help clarify the functions of invertase in reproductive processes

The association between high levels of both invertase activity and message levels with rapidly elongating styles (silk) in maize may have a twofold biological implication. First, invertase as one of the important constituents of sink strength (Morris and Arthur, 1984, Schaffer et al 1987) can provide an important avenue for sucrose cleavage Resulting hexoses can either be subsequently metabolized in

support of high respiratory rates and/or compartmentalized in vacuoles to maintain turgor for cell expansion. Second, invertase is demonstrated to contribute to an *in vitro* chemotropism of pearl millet pollen tubes toward stigmatic tissue through its production of glucose (Reger et al., 1992a, 1992b, 1993). Although other factors, such as calcium and an ovarian protein, are also important for the chemotropic response of pearl millet pollen tubes, our results were consistent with an invertase role in forming gradients of hexoses for pollen growth in maize. The message gradient of *Ivr1* and *Ivr2* abundance along the length of the silk (Figure 4-6) further supported the concept that glucose produced by invertase may be at least one key factor underlying the chemotropic response of pollen tubes, and their pathway towards the ovule.

Genes encoding carbohydrate metabolizing enzymes are regulated at the transcriptional level by sugar availability in yeast and vascular plants (Carlson, 1987; Schuster, 1989; Maas et al., 1990; Koch et al., 1992). Invertase, one of the only two enzymes known with a capacity to breakdown sucrose in vascular plants (Avigad, 1982), is also shown here to be regulated at the message level by sugar availability. Further, invertase gene subfamilies are found to be differentially expressed even within the same organ. The potential exists for these isozyme gene subfamilies to confer particular biological advantage through their presence in specific tissue at various stages of development and/or altered environmental conditions.

Photosynthesis and carbohydrate availability are often greatly reduced under environmental stresses, such as drought, flooding, severe cold and/or insect attack.

Because the *Ivr2* subfamily of genes are extremely sensitively to carbohydrate deprivation (Figures 4-8 4-9), the invertases encoded by these messages would be expected to contribute less to physiological process of importing cells under these circumstances.

However specific high priority developmental processes such as pollination and/or reproductive growth, once initiated, should ideally be less sensitive to changes in carbohydrate availability than is vegetative growth If the associated enzymes for sucrose metabolism are less sensitive to *de novo* down-regulation during sugar deprivation, then this could provide a mechanism for giving reproductive and other essential tissues "import priority" during stresses The *Ivr1* and *Ivr2* subgroup of genes could play contrasting roles in these instances much like those of *Sh1* and *Sus1* *Ivr1* related gene expression is strongly associated with reproductive structures (Figure 4-1) and is less markedly affected by carbohydrate deprivation (Figure 4-9) In contrast, the *Ivr2* subfamily of messages are widely distributed and clearly downregulated by in the absence of sugar supply The altered pattern of gene expression for *Ivr1* and *Ivr2* classes in response to carbohydrate deprivation may be an important adaptive strategy during different stresses, in which plant survival and/or reproduction could depend on the preservation of vital organs and/or tissues at the expense of others

This differential regulation of the two invertase subfamilies in response to sugar suggests that these genes and their respective enzymes may also have an important function in carbohydrate partitioning between sink and source tissues.

Under source-limited conditions, invertase involved in certain physiological processes could act to increase sink activity and stimulate assimilate translocation to these sinks to compete with others. Under normal growth conditions, assimilate levels are plentiful. Thus the *Ivr2* class of genes tend to be widely expressed in sink tissues and their gene products are abundant. This is especially evident in rapidly growing tissues, which is consistent with the concept that high activity of "soluble" invertase is usually associated with rapid tissue expansion (Glasziou and Gayler 1972, Giaquinta, 1979, Avigad, 1982, Hawker 1985, Schaffer et al., 1987). Invertase is considered to facilitate assimilate transportation from the site of phloem unloading to sink tissues by steepening the gradient of sucrose between source and sink (Shannon, 1968, Shannon et al, 1972, Shannon and Dougherty, 1972, Shannon et al., 1993). However soluble invertase can also promote cell elongation and/or rapid growth by hydrolyzing sucrose to hexoses, thereby providing osmotically active solutes and the osmotic pressure necessary to support growth (Kaufman et al. 1973, Schmulsig and Cosgrove 1988, 1990).

Gene responses to sugars in vascular plants have been known for some time (Rocha-Sosa et al., 1989; Salanoubat and Belliard, 1989, Muller-Rober et al., 1990, Maas et al., 1990, Koch et al., 1992). However the mechanism by which the sugar signal is sensed by plant genes is not clear. Our results (Figure 4-12) indicated that naturally occurring, metabolizable sugars, such as sucrose, D-glucose and fructose, meet the requirement for invertase responsiveness, although data shown here can not rule out other possibilities for certain non-metabolizable sugars.

Sadka et al (1994) propose that sugar modulates transcription of the soybean vegetative storage proteins and other sugar-inducible genes by using phosphate as a signal. In their model phosphate acts as a negative factor in these sugar-responsive genes. Carbohydrate activates these genes by accumulation of sugar phosphates and concomitant reduction of cellular phosphate levels. High phosphate levels relative to those of sugars are also found in starved sycamore cells (Rebeille et al., 1985).

Graham et al (1994) on the other hand, propose that not metabolism *per sé*, but the phosphorylation by hexokinase *per se* maybe signaling intracellular sugar responsiveness of gene expression. In their experiments, they demonstrate that 2 deoxyglucose and mannose, like glucose and fructose (which are phosphorylated by hexokinase but not further metabolized) specifically repress cucumber malate synthase and isocitrate lyase gene expression. However, 3-methylglucose, an analog of glucose that is not phosphorylated, does not result in repression of either malate synthase or isocitrate lyase.

Many of the genes involved in metabolic pathways are subject to regulation by the fluctuation of internal and external metabolites in multicellular vascular plants (Maas et al. 1990; Sheen, 1990; Koch et al., 1992; Graham et al., 1994; Sadka et al., 1994,). The metabolic regulation of gene expression should play a role of fundamental importance in maintaining an economical balance of the supply and demand of biomolecules in different organs of vascular plants. Metabolic control of specific gene expression now appears to be a widespread phenomenon, although the mechanism of signal transduction and response for different genes will not

necessarily be the same Environmental and developmental signals may also have contrasting influences, and depending on the role of the gene product, the sensitivity and degree of the response may also vary

CHAPTER 5
CYTOKININ MIMICS AND SUPERSEDES THE SUGAR INDUCIBILITY OF MAIZE INVERTASE FAMILY MEMBERS AND FACILITATES THEIR DIFFERENTIAL RESPONSIVENESS TO ABSCISIC ACID

Introduction

Plant growth regulators often affect many different aspects of plant growth and development. As an organism becomes more complex, communication between its different parts requires a signaling system with a progressively greater capacity to integrate distant messages. Hormonal responses belong to such a communication system (Lubbenga and Mennes, 1987). Much of the signalling in vascular plants is dependent upon a relatively complex array of hormonal signals.

Sucrose as the major form in which photoassimilates are transported, and as such plays a central and vital role in plant life (Avigad, 1982, Hawker 1985). Invertase is one of the two known enzymes which can initiate breakdown of this sucrose for further metabolism in vascular plants. It is thus considered a key enzyme for carbohydrate partitioning and utilization (Robbins, 1958, Glasziou and Gayler 1972, Avigad, 1982, Turgeon, 1989, Sturm and Chrispeels, 1990, Duke et al, 1991, Miller and Chourey 1992). Early studies of its activity showed that upregulation of capacity could be observed in response to abscisic acid, auxin, cytokinins, and/or gibberellic acid depending on species and conditions. The

diversity of systems examined included sugarcane stem segments (Sacher et al. 1963, Glasziou et al. 1966, Guyler and Glasziou, 1969) *Avena* stem segments (Kaufman et al. 1973), radish cotyledons (Howard and Witham, 1983) *Phaseolus vulgaris* seeds (Morris and Arthur, 1985), soybean seeds (Ackerson, 1985), *Citrus* leaves (Schaffer et al. 1987) tobacco crown gall cells (Weil and Rausch, 1990), etiolated *Pisum sativum* seedlings (Miyamoto et al., 1993), and shoots of dwarf pea (*Pisum sativum*) (Wu et al. 1993)

The present research was motivated by studies which showed that high invertase activity is usually associated with rapid growth. Action of this enzyme can provide tissues with not only substrates for their respiratory and synthetic demands (Morris and Arthur, 1984b, Schaffer et al., 1987) but also elevate turgor for cell expansion (Avigad, 1982, Schmalstig and Cosgrove, 1988, 1990)

Both cytokinins and ABA are reported to stimulate assimilate translocation from source to sink (Gersani and Kender, 1982, Howard and Witham, 1983, Heim et al., 1984, Schussler et al. 1984, Ackerson, 1985, Jones et al. 1986, Brokovec and Prochazka, 1992, Jones et al. 1992) ABA has been called a stress hormone since it accumulates during an array of stresses (Chen et al. 1983, Chen and Gusta, 1983, LaRosa et al. 1985, LaRosa et al. 1987, Davies and Zhang, 1991, Thomas et al. 1992) Both auxin and gibberellins stimulate cell enlargement, cell elongation and possibly phototropism and gravitropism (Davies, 1987, Kaufman and Song, 1987, Kim et al. 1993, Wu et al., 1993a, 1993b)

The hypotheses tested here are as follows: Invertase gene expression could be responsive to ABA (aiding osmoregulation), gibberellins and auxin (aiding gravitropism and phototropism), and/or cytokinins (aiding sink potential and/or symbiosis).

In this report, we demonstrate that in maize root tips both *Ivr1* and *Ivr2* expression for soluble acid invertase genes includes an unexpected, differential responsiveness to specific hormonal signals. These findings indicate that different invertase isozymes may have specialized functions in a diverse set of developmental and/or environmental processes.

Materials and Methods

Plant Material

Zea mays hybrid NK 508 was used for all experiments. Seeds were first emersed in 20 % Clorox for 30 min, followed by 30 min of continuous rinsing with water. Germination took place in the dark at 18 °C on two layers of moist 3 MM paper (Whatman, Inc., Clifton, NJ) in 17 x 26 cm glass pans. Air flowed continuously at 1 liter min^{-1} through each pan for the 6-day period, with 40% O_2 supplied during the final 24 hr before root tip excision. The moisture level was adjusted daily by applying mist and draining excess water. Root tips (ca 1 cm each) were excised under a sterile transfer hood.

Experimental Conditions

Experimental treatments were as described by Koch et al (1992) Approximately 100 root tips (~ 500 mg) were used for each experimental treatment. Excised root tips were incubated in the dark at 18 °C for 6 to 48 hr in Whites' medium plus 0.5% glucose, either with or without specific supplemental plant growth regulators (ABA, A1049 GA, G7645, Kinetin, K0753 IAA, I2886 all from Sigma) Each group of root tips was agitated at 120 cycles per minute in a 125 ml side-arm Erlenmeyer flask with 50 ml of sterile media. Airflow (40% O_2) through air stones in each flask was maintained at 250 ml min^{-1} throughout the incubations

RNA Isolation and Blot Analysis

Root tip samples were rinsed twice in sterile water, blotted dry weighed, and frozen in liquid N_2 Samples were ground into fine power in liquid N_2 and total RNA was extracted as per McCarty (1986) RNA was quantified spectrophotometrically (Sambrook et al 1989)

Total RNA was separated by electrophoresis in 1 % agarose gels containing formaldehyde (Thomas, 1980) blotted to a nylon membrane and fixed by baking and/or UV treatment (Sambrook et al , 1989) Filters were hybridized at 65 °C in a solution containing 7 % SDS 250 mM Na_2HPO_4 pH 7 2, 1 % BSA (Church and

Gilbert, 1984) Maize *Ivr1* and *Ivr2* invertase cDNA clones were radiolabeled by random primer No cross-reactivity between *Ivr1* and *Ivr2* gene probes was observed when hybridizations were conducted at high stringency (data not shown) Blots were washed as described by Church and Gilbert (1984) exposed to X-ray film with intensifying screens at -80 °C

Enzyme Extraction

Soluble invertase was extracted as per Duke et al (1991) Frozen tissue samples were ground to a fine powder in liquid N_2 using a mortar and pestle Frozen powder was transferred to a second mortar containing ice-cold 200 mM HEPES buffer (pH 7.5) with 1 mM DTT 5mM $MgCl_2$, 1 mM EGTA and 10%(w/w) PVPP One ml of extraction buffer was used for every 100 mg of tissue fresh weight Buffered extract was centrifuged at 15 000 x g for 10 min to sediment particulate matter Pellets were saved for salt solubilized particulate invertase extraction Supernatant was dialyzed (50,000 MW cutoff) at 4 °C for 24 hr against extraction buffer diluted 1 40 (MW cutoff for dialysis was selected to allow escape of proteinaceous invertase inhibitors [Jaynes and Nelson, 1971b]) Buffer was changed twice Soluble dialyzed extract was centrifuged again at 15 000 x g for 10 min Supernatant was used for soluble invertase assays as described below

Insoluble invertase was extracted as described by Doehlert and Felker (1987) Pellets remaining from the above step were washed three times by resuspending in

extraction buffer and centrifuging at 15,000 x g for 10 min. Salt solubilized particulate invertase was extracted by resuspending the pellet in extraction buffer containing 1 M NaCl. Solubilized particulate invertase was recovered in supernatant following centrifugation at 15,000 x g for 10 min. Supernatant was used for insoluble invertase assays as described below.

Enzyme Assay

Both soluble and salt solubilized invertase activities were assayed for 15 to 30 min at 37 °C in a mixture containing 100 mM Na-acetate (pH 4.5) and 100 mM sucrose in a final volume of 500 µl. Activity was determined by measuring reducing sugars with the method of Nelson (1944) and Somogyi (1951).

Results

Cytokinin (5 μM kinetin) exposure evoked a positive response at the level of message abundance for both the *Ivr1* and *Ivr2* gene subfamilies as well as at the level of total soluble acid invertase enzyme activity (Figure 5-1). The responses of both gene subfamilies to kinetin were similar (elevated 2.5-fold) and maximal at 5 μM (tested range from 1 to 200 μM; data not shown). The same treatment resulted in an 1.5 fold elevation of total soluble acid invertase activity within these 24 hr experiments (Figure 5-1 B).

The positive responses of the *Ivr1* and *Ivr2* gene subfamilies to exogenous cytokinin were also evident when the excised root tips were depleted of carbohydrates (Figure 5-1). Kinetin mimicked and superseded the sugar-enhanced expression of both the *Ivr1* and *Ivr2* classes of invertase. The fact that kinetin could replace and override carbohydrate supply in this respect was also evident at the level of total soluble acid invertase activity (Figure 5-1 B).

When root tips were incubated with exogenous ABA (50 μM [which gives the maximal response range from 1 to 200 μM; data not shown]) for 24 hr, levels of message encoding both the *Ivr1* and *Ivr2* invertase subfamilies were elevated 1.5-fold and 3-fold respectively (Figure 5-2 A). The *Ivr1* subgroup responded less markedly than did its *Ivr2* counterpart. Although maximal responses to exogenous ABA were observed at 50 μM for transcripts from both the *Ivr1* and *Ivr2* invertase

classes, only minimal affects were evident at the level of total soluble acid invertase activity (Figure 5-2 B)

The fact that the elevated levels of mRNA encoding *Ivr1* and *Ivr2* subfamilies did not result in altered enzyme activity raised the question of whether or not ABA upregulated enzyme activity The possibility remained that the responses of soluble invertases to ABA at the translational level might be enhanced by cytokinins as had been observed for sugar modulated expression of these genes

To address this question, ABA incubations were supplemented with low levels (5 µM) of cytokinin In figure 5-2, total soluble acid invertase activity was higher when both ABA and kinetin were added to root tips than it was with kinetin alone (Figure 5-2 B) The responses of *Ivr1* type genes to ABA were overridden by kinetin (Figure 5 2 A) *Ivr1* related message levels were the same when excised root tips were treated with either kinetin alone or the combination of kinetin and ABA. Levels of mRNA from the *Ivr2* subgroup, on the other hand were significantly higher when both ABA and kinetin were applied to the incubation solution than when ABA or kinetin were present alone. Elevation of the *Ivr2* type message levels was additive for each single treatment.

The responses of both *Ivr1* and *Ivr2* classes of invertase genes were much less markedly affected by exogenous GA and/or IAA than they were by ABA and/or kinetin (Figure 5-3 A) No changes were evident in activity of total soluble acid invertase in response to root tip incubations with either GA or IAA (Figure 5-3 B).

Figure 5-1 Abundance of mRNA from the *Ivr1* and *Ivr2* subfamilies of soluble acid invertase and activity of total soluble acid invertase in maize root tips incubated for 24 hr in White's basal salts medium supplemented either with (+G) or without (-G) 0.5% glucose and either with (+K) or without (-K) 5 μM kinetin. A, RNA gel blots with equal amounts (10 μg) of total RNA from above tissues were probed with ^{32}P labeled *Ivr1* or *Ivr2* representing the two subfamilies of maize soluble acid invertase. Blots were exposed to X-ray film for 24 or 12 hr respectively. B. Total soluble acid invertase activity from the above samples. Insoluble invertase activity (not shown) was consistently ca 10-fold less than that in the soluble fraction of maize root tips. Values for RNA/protein recovery were ca 0.15 (±0.06) with variability independent of +K or -K treatments.

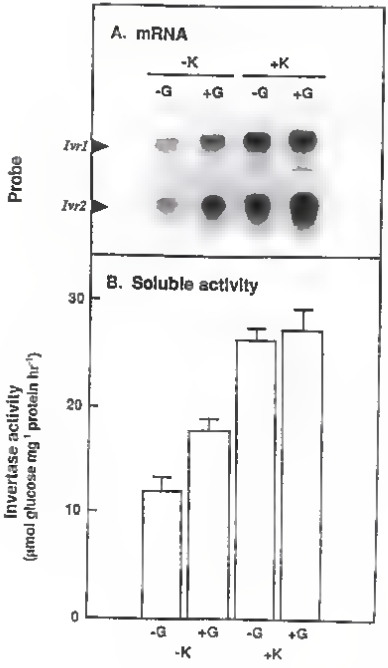

A. mRNA
-K
+K
-G
+G
-G
+G
Probe
Ivr1
Ivr2
B. Soluble activity
Invertase activity
(μmol glucose mg⁻¹ protein hr⁻¹)
30
20
10
0
-G
+G
-G
+G
-K
+K

Figure 5-2 Abundance of mRNA from the *Ivr1* and *Ivr2* subfamilies of soluble acid invertase and activity of total soluble acid invertase in maize root tips incubated for 24 hr in White's basal salts medium supplemented with 0.5% glucose (all +G), either with (+K) or without (-K) 5 μM kinetin, either with (+ABA) or without (-ABA) abscisic acid (50 μM). A, RNA gel blots with equal amounts (10 μg) of total RNA from above tissues were probed with ^{32}P labeled *Ivr1* or *Ivr2* representing the two subfamilies of maize soluble acid invertase. Blots were exposed to X-ray film for 24 or 12 hr respectively. B, Total soluble acid invertase activity from the above samples. Insoluble invertase activity (not shown) was consistently ca. 10-fold less than that in the soluble fraction of maize root tips. Values for RNA/protein recovery were ca. 0.20 (±0.07) with variability independent of +K or +ABA treatments.

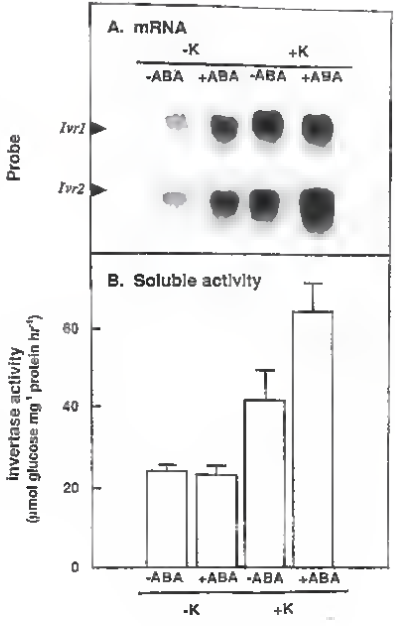
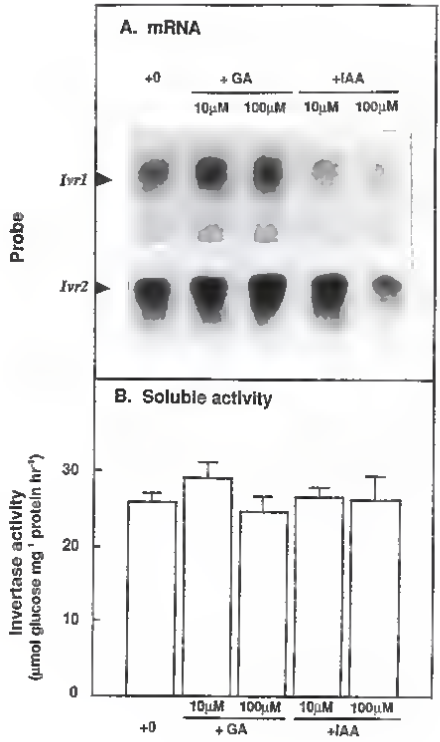

A. mRNA
-K
+K
-ABA
+ABA
-ABA
+ABA
Probe
Ivr1
Ivr2
B. Soluble activity
invertase activity
(μmol glucose mg⁻¹ protein hr⁻¹)
60
40
20
0
-ABA
+ABA
-ABA
+ABA
-K
+K

Figure 5 3 Abundance of mRNA from the *Ivr1* and *Ivr2* subfamilies of soluble acid invertase and activity of total soluble acid invertase in maize root tips incubated for 24 hr in White s basal salts medium supplemented with 0 5% glucose (all +G) alone (+0) or supplemented with either gibberellic acid (+GA) or auxin (+IAA) A, RNA gel blots with equal amounts (10 µg) of total RNA from above tissues were probed with ^{32}P labeled *Ivr1* or *Ivr2* representing the two subfamilies of maize soluble acid invertase Blots were exposed to X ray film for 24 or 12 hr respectively B. Total soluble acid invertase activity from the above samples Insoluble invertase activity (not shown) was consistently ca 10 fold less than that in the soluble fraction of maize root tips. Values for RNA/protein recovery were ca 0 23 (±0 12) with variability independent of +GA or +IAA treatments.


A. mRNA
+0
+ GA
+IAA
10μM
100μM
10μM
100μM
Probe
Ivr1
Ivr2
B. Soluble activity
Invertase activity
(μmol glucose mg⁻¹ protein hr⁻¹)
30
20
10
0
10μM
100μM
10μM
100μM
+0
+ GA
+IAA

Discussion

The significance of findings presented here is that they indicate different environmental and/or developmental signals can regulate the same gene expression through common and/or different pathways. Thus, the same enzyme reaction could play multiple roles under various conditions. Sugar-modulated genes are also responsive to plant growth regulators. These responses provide a potential mechanism by which import organs may adjust their sucrose-metabolizing capacity to altered environment and/or developmental stages.

Results shown here also indicate that cytokinin has a positive effect on the invertase gene system and that both mRNA abundance and soluble acid enzyme activity are up-regulated by exogenous cytokinin (Figure 5-1). Cytokinin is reported to stimulate translocation of photosynthate from source leaves to cytokinin-treated areas thus increasing the sink capacity of importing bean leaves (Gersani and Kender 1982), radish cotyledons (Howard and Witham 1983), winter wheat grains (Borkovec and Procharka, 1992) and developing maize kernels (Jones et al., 1992).

During leaf development of snap bean (*Phaseolus vulgaris L.*) and *Citrus*, soluble acid invertase activity is correlated well with leaf expansion. In contrast, both insoluble invertase and sucrose synthase activities are low and show little change during leaf development (Morris and Arthur 1984, Schaffer et al., 1987). The authors suggest that soluble acid invertase activity is the primary enzyme responsible for sucrose catabolism in the expanding bean and citrus leaves. Its

activity is considered to be the primary determinant of sink strength in these systems. In addition, early development of maize kernels (Tsai et al , 1970) and snap bean pods (Sung et al , 1994) takes place with soluble invertase predominating whereas sucrose synthase activity in both cases is below level of detection Cytokinins stimulate soluble acid invertase gene expression which subsequently increases sink potential

Mycorrhizal fungi and/or rhizobia can only metabolize hexoses. Cytokinins and/or cytokinin-like substances are reported to be synthesized in mycorrhizal fungi (Miller, 1967 Crafts and Miller 1974, Ng et al., 1982) and *Bradyrhizobium japonicum* (Shortevant and Taller, 1989) Allen at al (1980) report that cytokinin level increases in the host plant after infection by vesicular-arbuscular mycorrhizae Cytokinins originating from mycorrhizal fungi and/or rhizobia act to increase the sink capacity through elevating invertase expression from the host plant. The present work therefore indicates that cytokinin like substances from fungal and/or rhizobial symbionts could also act through stimulation of invertase

Figure 5-1 suggested, first, that cytokinin and sugar enhanced the accumulation of invertase message level independently Both *Ivr1* and *Ivr2* mRNA levels were elevated ca 2 5 fold by cytokinin regardless of exogenous carbohydrate supply Sugar alone was unable to stimulate invertase expression to the same degree as was cytokinin. Second, cytokinin alone was sufficient to promote invertase enzyme activity to the highest level after 24 hr incubation in this system (Figure 5 1B)

The biological significance of the findings presented here is twofold. First, under stress conditions severe enough to limit photosynthesis, cytokinins still could maintain sink potential of specific regions by preserving invertase activity for later recovery of growth. Second, cytokinins could possibly initiate and/or enhance the capacity for sucrose import and utilization in a given structure simply by stimulating invertase expression. Jones et al. (1992) found that cytokinin levels increase dramatically (as much as 400-fold) during the early stages of maize kernel development and decline subsequently. They suggest that the ultimate capacity for sucrose import into kernels is established during this first portion of development, and further, that *de novo* biosynthesis of cytokinins within kernels plays a role in this process through regulation of endosperm and/or nucellar development.

The responsiveness of invertase gene expression to exogenous ABA may also have a distinct biological relevance (Figure 5-2). ABA is considered a stress hormone due to its accumulation and action during many such conditions (Cheo et al., 1983; Chen and Gusta, 1983; LaRosa et al., 1985; LaRosa et al., 1987; Davies and Zhang, 1991; Thomas et al., 1992). Thus, ABA could enhance the capacity of a plant to acclimate to different stresses, such as freezing, drought and high salt, by inducing invertase expression. This in turn has the capacity to aid adjustment of osmotic potential.

ABA is also reported to stimulate transport of photosynthate towards developing seeds in a number of species (Hein et al., 1984; Sebussier et al., 1984; Ackerson, 1985; Jones et al., 1986; Borkovec and Prochazka, 1992). ABA could

facilitate this by elevating invertase activity and enhancing early expansion. Ackerson (1985) found that soluble acid invertase activity in developing soybean reproductive structures is correlated well with endogenous ABA levels. Sung et al. (1994) also observed that invertase predominates during early development of pods. Data here indicated that both *Ivr1* and *Ivr2* mRNA levels were elevated after exogenous applications of ABA, which supported the above hypotheses (Figure 5-2 A).

Total soluble acid invertase activity, however, remained unchanged (Figure 5-2 B). At this point, the mechanism by which exogenous ABA treatment could elevate invertase message levels without affecting enzyme activity remains unclear. Simultaneous presence of cytokinin appeared to be necessary before message-level responses could be transduced at the level of enzyme activity (Figure 5-2).

In general, cytokinins are most abundant in young organs (seeds, fruits and leaves) and in root tips (Salisbury and Ross, 1992). Endogenous cytokinin levels are similar in the presence or absence of salt stress in *Mesembryanthemum crystallinum* (Thomas et al., 1992). During early development of soybean pods, winter wheat and maize kernels, both endogenous cytokinin and ABA levels are high (Ackerson, 1985; Borkovec and Prochazka, 1992; Jones et al., 1992). Thus, it is possible that there are sufficient levels of endogenous cytokinins to allow the ABA responsiveness of one or more soluble invertase genes to be extended to the level of enzyme activity. This could be particularly significant to the physiology of young reproductive structures under stresses and/or during their earliest development.

A further implication here lies in the response of drought-stressed roots. The ABA effect on osmotic adjustment would be nominal to non-existent unless cytokinins were present. Because cytokinins are normally carried away in the xylem stream, a rapidly transpiring plant may have less cytokinins build up in the root tips than one with stomata closed during drought stress. Further extension of this scenario could potentially include root to shoot signalling via cytokinin flow and subsequent effect on shoot soluble invertase (Davies and Zhang, 1991).

Action of soluble invertase has been implicated in gravitropism (Kaufman and Song, 1987; Kim et al., 1993; Wu et al., 1993a, 1993b) and may also be involved in some aspects of phototropism (Davies, 1987; Kaufman and Song, 1987). Elevation of osmotic potential for cell elongation could readily result from invertase hydrolysis of sucrose within the vacuole, and the asymmetry of this process across a stem is consistent with the involvement of similarly distributed plant growth regulators in action of invertase. This suggestion is supported by the observation that glucose injection into dwarf pea shoots mimics the effect of GA on cell elongation (Broughton and McComb, 1971).

For this reason, altered expression of *Ivr1* and/or *Ivr2* was expected in response to treatment with auxin and/or GA. However, neither the invertase message levels nor assayed enzyme activity were markedly affected by addition of exogenous auxin and gibberellic acid to the root tip system used here (Figure 5-3). One explanation might be as follows. Hormones often have pleiotropic effects, i.e. different types of target cells all respond to the same set of signals, but in a different

way. In many cases, these types of target cells have similar perception-and-transduction mechanisms, but the molecular programs which are elicited by these mechanisms are different (Libbenga and Mennes 1987). Gravitropism in shoots is brought about by auxin-induced elongation on the long side. In roots, however, auxin does not induce, but rather inhibits elongation. In figure 5.3, the abundance of both the *Ivr1* and *Ivr2* type messages was downregulated in response to IAA treatment, however the total soluble acid invertase activity remained relatively constant. One explanation may be the apparent longevity of some invertase proteins.

A need for greater activity of invertase has been implicated in the establishment and enhancement of the capacity for sucrose import (Morris and Arthur, 1984, Hanft and Jones 1986a, Schaffer 1987). Phytohormone modulation of invertase genes could effectively regulate the processes associated with phloem unloading and expansion sink potential (Hess et al. 1984, Schussler et al. 1984, Ackerson 1985, Jones et al., 1986, Borkovec and Prochazka, 1992, Jones et al., 1992).

Genes specifying plant growth regulator biosynthesis have been identified in phytopathogens (Miller 1967, Crafts and Miller 1974, Allen et al. 1980, Ng et al 1982, Morris, 1986, Weil and Rausch, 1990). Plant hormone regulation of invertase expression could facilitate its enhancement, in instances such as establishment of symbioses, by providing hexoses for those fungi unable to metabolize sucrose

CHAPTER 6
SUMMARY AND CONCLUSIONS

Sucrose, as the principle form for assimilate transport, plays a central vital role in plant growth and development. Invertase catalyzes one of the only two enzymatic reactions known capable of breaking down sucrose for further metabolism in vascular plants, and is thus considered an essential enzyme in the control of carbohydrate partitioning and utilization. High invertase activity is usually associated with rapid growth. Soluble forms of this enzyme can be especially important to cell expansion through generation of hexoses and their associated osmotic potential. The purpose of this study was to test hypotheses relating expression of soluble invertases to specific developmental processes, carbohydrate responses, and/or changes in plant growth regulators.

The first step toward this end was accomplished through isolation of five cDNA and one genomic clones encoding soluble invertase isozymes in maize. These were obtained by screening maize root tip and a seedling genomic libraries with a heterologous tomato soluble invertase (Klann et al. 1992). The deduced amino acid sequences showed significant identities to previously characterized invertases. Each of the highly conserved domains existing in other invertases were

also present in the maize clones examined here. Further these maize invertases shared considerably greater identity to soluble invertases than to their cell-wall-bound counterparts. The maize genes examined here thus probably encode soluble enzymes. This conclusion was also supported by a strong correlation between the transcript levels of both *Ivr1* and *Ivr2* subfamilies relative to the total soluble invertase activities in an array of maize tissues and/or developmental stages.

Ivr1 and *Ivr2* subfamilies of soluble invertase were mapped to two and four different loci, respectively, each on a different chromosome (in collaboration with Scott Wright, Genetic Linkage). One of the *Ivr1* genes mapped to a region near the *miniature* mutation in maize (kernels known to lack insoluble invertase). However, further analysis showed both *Ivr1* and *Ivr2* message to be present in this mutant line as well as wild-type levels of soluble invertase activity. Again this evidence supports a soluble invertase identity for the invertase family of genes isolated here.

The maize genomic DNA for *Ivr1* was found to be organized into seven exons and six introns. The second exon is only 9 nucleotides long, but encodes the highly conserved domain found in all the invertases (NDPNG, the β-fructosidase motif). This 9 bp exon is probably the smallest exon thus far identified in the plant genome (M. Schuler, personal communication).

The expression of two classes of maize invertase (*Ivr1* and *Ivr2*) was further characterized to test the hypothesis that specific genes might be associated with different developmental stages and/or enlargement of key sets of cells. The two invertase subgroups were differentially expressed in roots, sink leaves, young

kernels, immature and mature anthers, pollen and silk. A comparison between transcript and enzyme activity was again consistent with both clones encoding soluble acid invertases. The spatial and temporal patterns of expression for the two invertase subfamilies as well as the contrast between them, demonstrated a close association between soluble invertases and changes in silk during pollination and in kernels immediately after fertilization.

Maize root tips in 24 hr culture were used to quantify the extent to which soluble invertase (at the message and enzyme levels) responded to sugar and specific developmental/environmental signals. In this system, the abundance of mRNA levels from both classes was upregulated whenever a source of exogenous, readily metabolizable, sugars was made available. The extent of response differed from one invertase subgroup to another, however. The *Ivr2* type genes showed greater sensitivity to carbohydrate deprivation. The differential responsiveness of invertase gene subfamilies to carbohydrate availability provided a potential mechanism for different isozyme gene subgroups to predominate in various tissues, developmental stages, and/or altered environmental conditions.

Several lines of investigation have suggested invertase gene expression may be responsive to cytokinins (thus enhancing potential for sucrose metabolism, import and/or symbiosis), water deficit and/or ABA (aiding osmotic alteration), and gibberellin or auxin (aiding gravitropism and/or phototropism). Kinetin treatments (5 μM) increased both message levels (*Ivr1* and *Ivr2*) and elevated total soluble acid invertase activity. Kinetin alone replaced and superseded the carbohydrate

enhancement for invertase expression. Although mRNA abundance was enhanced by exogenous ABA, no significant change was evident in total soluble acid invertase activity, when ABA alone was added. In contrast, treatments combining cytokinin with ABA, resulted in maximal responses at both transcript levels and enzyme activity levels. Simultaneous presence of ABA and cytokinin appeared to be necessary before message-level responses could be transduced at the level of enzyme activity in this case. Neither the invertase message levels nor assayed enzyme activity were markedly affected by additions of gibberellic acid or auxin. These findings provide a potential mechanism by which importing organs may adjust their sucrose-metabolizing capacity to altered environment and/or developmental stages.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy

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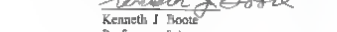

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